PRACTICAL HÆMATOLOGY

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By

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AUTHOR'S PREFACE

This little book on Practical Hæmatology had its origin in notes on hæmatological technique and interpretation prepared for students taking a one year course for the London University Diploma in Clinical Pathology. It does not claim to be a reference book to Laboratory Methods, and in most instances one technique only for a particular procedure has been described. Its scope is a restricted and arbitrary one, and I have deliberately omitted subjects which seem to me to belong to morphology or medicine rather than to laboratory technique. The methods outlined are, however, those of which I have had experience, and they are in routine use in the Hæmatology Laboratory of the Postgraduate Medical School of London

I think every laboratory worker feels that the burden of routine investigation is often too great and that too much time and energy are expended on the normal, with the result that abnormal conditions are not properly investigated and general technical standards are lowered. It is clearly desirable to make the most effective use of bench space and personnel, and for this reason I have included some notes and opinions on the relative value of hæmatological investigations and the order in which it seems to me that they should be undertaken.

I feel it is most important that all those concerned in laboratory work should understand as far as possible what is the significance of the tests they carry out. I have, therefore, added notes here and there which are intended to make more intelligible some of the procedures described These notes are perhaps intended more for technicians than for those with medical training. In fact all through I have kept the needs of technicians in mind, and I hope this book will help to make more interesting the essential work they do.

I should like to thank Professor J. H. Dible, Dr I. Doniach, Professor E. J. King, Dr D. L. Mollin, Dr. P. L. Mollison, Mr. L. H Turnbull, Dr. J. C. White and Dr. I D. P. Wootton for valuable advice and criticism, and Mrs M. E. Harvey for secretarial assistance

TABLES

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Chapter 1

BLOOD SAMPLES FOR CLINICAL HÆMATOLOGY

Collection of blood samples. Normal values. Physiological variation.

COLLECTION OF BLOOD SAMPLES

Venous blood is to be preferred for most hæmatological examinations, but peripheral samples can be almost as satisfactory for some purposes if a free flow of blood is obtained.

Venous Blood

This should be taken from an antecubital vein into a dry syringe using a relatively wide bore needle, or by means of a similar needle to which 2 in of rubber tubing have been attached (see appendix 5) Ideally, congestion should be completely avoided, in practice a tourniquet is usually necessary—it can often be loosened once the needle has been inserted into the vein. It is likely that complete stasis for as little as 30 seconds causes some hæmoconcentration (1). The piston of the syringe should be withdrawn slowly and no attempt made to withdraw blood faster than the vein is filling. The blood when withdrawn should be promptly and thoroughly, but gently, mixed with the anticoagulant. Hæmolysis can be avoided or minimized by using clean apparatus, withdrawing the blood slowly, not using fine needles, delivering the blood gently into the receiver and avoiding frothing during the withdrawal of the blood and subsequent mixing with the anticoagulant.

Blood collected in order to obtain serum is conveniently delivered into sterile tubes or capped bottles and allowed to clot undisturbed at room temperature. When the blood has firmly clotted and the clot has commenced to retract, the sample should be placed in a refrigerator overnight at 2–5°C.,

pathological forms may be simulated. Mistakes may be made unless this type of change is appreciated.

Heparin at a concentration of 0.1 mg. to 0.2 mg. per ml. of blood is a good anticoagulant. It does not alter corpuscular size and is the best anticoagulant to use if it is important to avoid hæmolysis. Heparinized blood, however, should not be used for leucocyte counts for some of the cells may clump together.

oxalate (crystals of which may be seen in oxalated blood) or bound to the citrate molecule in an unionized form Heparin inhibits coagulation in a different way, it is thought to have the power of neutralizing thrombin Ammonium and potassium oxalates are poisonous and are for laboratory use only Sodium citrate and heparin can be used to render blood incoagulable before transfusion.

Storage of Blood before Estimations are Performed

Certain changes take place when blood is allowed to stand *in vitro*; these can be minimized by keeping the samples in the refrigerator at a temperature of 2–5°C The red corpuscles start to swell, the corpuscular volume tends to increase, osmotic fragility and prothrombin time slowly increase and the sedimentation rate decreases: the leucocytes gradually autolyse (2) Hæmoglobin remains unchanged for days. These changes, however, take place slowly, and for most purposes blood may be safely allowed to stand overnight in a refrigerator if precautions against freezing are taken A rule should be made, however, to count leucocytes as soon as possible and perform sedimentation rates within six hours of collection The advisability of making films at once has already been mentioned. The importance of effective mixing of blood after collection, particularly if it has been stored and is cold and viscous, cannot be over-emphasized. The tube if cold should first be allowed to warm up to room temperature, then mixed by rotation and tilting for at least two minutes. This difficulty of mixing stored blood is a strong point in favour of performing blood counts without delay.

so that there may be time for retraction to be complete and little likelihood of the growth of bacteria. If the clot when firmly formed shows no sign of retraction, it may be gently detached from the walls of the container by means of a platinum wire or a sealed drawn out Pasteur pipette. If it is roughly treated, hæmolysis is certain to follow. When serum is required without delay, clotting and retraction may be hastened by placing the sample in an incubator or water bath at 37°C., or by defibrinating the sample. Defibrination is simply performed by placing the blood in a receiver such as a conical flask containing a central glass rod on to which small pieces of glass capillary have been fused (see appendix 3). The blood is whisked around the central rod by gentle rotation of the flask and clotting is usually complete within five minutes. The fibrin collects upon the central rod. When this has happened, the defibrinated blood may be centrifuged, and serum obtained quickly and in relatively large volumes. Blood defibrinated in this way should not undergo any appreciable degree of hæmolysis

Anticoagulants

The *ammonium and potassium oxalate mixture of Heller and Paul* has been recommended by Wintrobe and is widely used. At a concentration of 2 mg. of the mixed salts (six parts of ammonium oxalate to four parts of potassium oxalate) to 1 ml. of blood, the corpuscular volume is unaltered and little hæmolysis caused. Such blood may be used for hæmatocrit and hæmoglobin estimation, and for red cell and leucocyte counting, and the plasma for the estimation of bilirubin and prothrombin time. Films are satisfactory if made without delay. If the blood is allowed to stand before films are made, definite changes take place in the morphology of the corpuscles. The red cells start to crenate, but more important are the changes in the leucocytes. Within an hour abnormalities become obvious and are progressive. The cytoplasm of the granulocytes becomes vacuolated and ingested oxalate crystals may be seen, and the nuclei tend to become pyknotic. The nuclei of lymphocytes and monocytes undergo bizarre changes in shape; lobation or budding takes place and

Differences between Peripheral ("Capillary") and Venous Blood

It is not quite clear whether the packed cell volume (hæmatocrit), red cell count and hæmoglobin content of venous blood and peripheral blood are the same, even if the

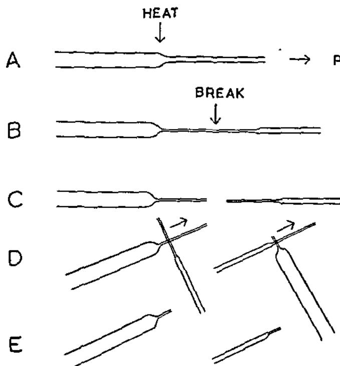


FIG 1 MAKING GLASS CAPILLARY PRICKERS

latter is freely flowing. Although the results of Price-Jones, Vaughan and Goddard (9) did not reveal any certain differences in normal adults, this may not be always true. Strauss

Peripheral ("Capillary") Blood. This is best obtained from the ear of an adult or from the heel of an infant. As already mentioned a free flow of blood is essential, and only the very gentlest squeezing is permissible; ideally large drops of blood should exude slowly but spontaneously. If it is necessary to squeeze firmly in order to obtain blood, the results are generally unreliable. If the poor flow is due to the part being cold or cyanosed, abnormally high figures for red cell counts and hæmoglobin and for leucocyte counts are usually obtained.

The ear is preferable to the finger; it is less sensitive, and generally bleeds well. If cold, the lobe must be rubbed with lint until warm. It is then pricked to a depth of 2–3 mm. with a needle (Hagedorn) or glass capillary pricker.* Wipe off the first few drops and collect the sample when the blood is flowing spontaneously, usually in about half a minute. There seems to be little risk of introducing organisms into the ear if a sterile needle or glass capillary is used. The practice of pricking successive patients with the same needle is to be deprecated, even if the needle is placed in spirit between each puncture. The risk of transmitting "serum jaundice" in this way cannot altogether be neglected. If the needle is repeatedly "flamed," it soon becomes blunt For these reasons it is recommended that a separate glass capillary pricker be used for each patient. These are made from wide glass capillaries partially pulled out (see *Fig.* 1).

With a little practice, it is possible to break them so that the cut end is smooth and vertical. The pricker is placed against the most dependent part of the lobe of the ear which is then punctured to a depth of 2–3 mm. by gentle rotation of the pricker.

Heel Blood. Satisfactory samples can be obtained by a deep puncture, but only if the heel is really warm—it may be necessary to bathe it in hot water

[illegible] were described [illegible]
student.

additional references are listed later when the relevant technical methods are considered. An attempt has been made to maintain a moderate perspective in respect of "normal ranges," particularly where large discrepancies exist between the findings of different authors. The figures given are believed to cover at least 95 per cent of healthy subjects. Statistically speaking, a value at the edge of a supposed normal range is naturally more likely to be abnormal than normal.

PHYSIOLOGICAL VARIATIONS IN BLOOD VALUES

Physiological Variation in Hæmoglobin, Hæmatocrit and Red Cell Counts (4, 5)

It is well known that there is considerable variation in the red cell count and hæmoglobin at different periods of life. At birth the hæmoglobin content of the blood is higher than at any period subsequently (see Table I). After the immediate post-natal period the hæmoglobin level falls fairly steeply to a minimum of about 11 g. at about the 5th to 18th month, but there is considerable variation from child to child. The red cell count falls less steeply and the corpuscles become hypochromic. Both hæmoglobin and red cell count then gradually rise to almost the adult level by the time puberty is reached; thereafter the levels in women tend to be significantly lower than those of men.

The values found in the immediate post-natal period vary

Probably it is the cessation of pulsations of the cord as well as the uterine contractions which cause the blood contained in the placenta to re-enter the infant's circulation.

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and Burchenal (13) in a survey of 80 patients under treatment for pernicious anæmia found that their red cell counts and hæmoglobin levels averaged 5 per cent higher in "capillary" than in venous blood, a difference which was statistically significant. In infants, too, similar differences probably exist (11), at least in the neonatal period.

It is likely that freely flowing blood obtained by skin puncture is more nearly arteriolar in composition rather than capillary. Indeed, the hæmatocrit value, red cell count and hæmoglobin content of true capillary blood is probably significantly less than that of venous blood [Gibson *et al.* (3)]. The platelet count appears to be higher in venous blood, but the leucocyte levels are probably identical if counted in freely flowing peripheral blood—if the ear is cold, the leucocyte count may be raised above the venous level. The osmotic fragility of venous blood is significantly greater than that of peripheral blood, due probably to the reduced oxygen tension in the venous sample.

Satisfactory blood films can be made from venous blood. *As already mentioned they must be made without delay, for* the leucocytes will undergo degenerative changes on standing. Often it is more satisfactory to make films separately from an ear prick.

NORMAL VALUES IN HÆMATOLOGY

It is extremely difficult to state the limits of normality in respect of blood findings; the observed ranges are considerable, and age and sex determine important differences. In addition, variation in technique may explain differences between some observers' figures; particularly is this true of counting platelets and of estimating coagulation time.

The limits of health itself are ill defined, and the normal and abnormal undoubtedly overlap; for instance, a value well within the recognized "normal range" may be definitely pathological in a particular subject, e.g. a total leucocyte count of 10,000 per c.mm. is abnormal for a man whose count usually ranges between 4000 and 6000 per c.mm.

The data given in Table I comes from various sources (1-9).

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Probably it is the cessation of pulsations of the cord as well as the uterine contractions which cause the blood contained in the placenta to re-enter the infant's circulation.

In addition to the permanent effects of age and sex, there seem to be transient fluctuations the significance of which is often difficult to assess. Muscular activity, if at all severe, unquestionably will raise the levels of both red cells and hæmoglobin, presumably largely due to a redistribution of cells within the circulation and/or loss of plasma; increases amounting to 0.5

Table I—NO

Variatio

Red blood cells	
Men	4.5–6.5 millions per c.mm.
Women	3.9–5.6 ,, ,,
Infants at birth (cord blood)	4.0–5.6* ,, ,,
Children, 1 year	4.5 (average) ,, ,,
Children, 10 years	4.7 (average) ,, ,,
Hæmoglobin	
Men	13.5 g.–18.0 g. per 100 ml.
Women	11.5 g.–16.4 g. ,,
Infants at birth (cord blood)	14.0 g.–19.0 g.* ,,
Children, 1 year	11.2 g. (average) ,,
Children, 10 years	12.9 g. (average) ,,
Packed cell volume	
Men	40–54%
Women	36–47%
Infants at birth (cord blood)	44–62%*
Children, 1 year	35% (average)
Children, 10 years	37.5% (average)
***Mean corpuscular volume* (M.C.V.)**	
Adults	76–96 c.μ.
***Mean corpuscular hæmoglobin* (M.C.H.)**	
Adults	27–32 μμg.
***Mean corpuscular hæmoglobin concentration* (M.C.H. conc.)**	
Adults	32–36%
***Mean corpuscular diameter* (dry films)**	
Adults	6.6–7.7 μ
Reticulocytes	
Adults	0.2–2%
Infants at birth	2–6%*

* Unpublished observations

MAL VALUES
in Health

Leucocytes (90% subjects)

Adults	4000–10,000 per c.mm
Birth	10,000–25,000 ,, ,,
1 year	6000–18,000 ,, ,,
Childhood (4–7 years)	6000–15,000 ,, ,,
Childhood (8–12 years)	4500–13,500 ,, ,,

Differential leucocyte count

Adults	Polymorphs.	40–75%	2500–7500 per c.mm.
	Lymphocytes	20–30%	1500–3500 ,, ,,
	Monocytes.	2–10%	200–800 ,, ,,
	Eosinophils.	1–6%	200–400 ,, ,,
	Basophils	1%	15–100 ,, ,,

Platelets 150,000–350,000 per c.mm

Bleeding Time (Duke's method) 1–5 min.

Coagulation Time (Lee and White's method, 37°C) 4–10 min

Osmotic fragility

1. Dacie and Vaughan's method

0 26–0 34% NaCl	"complete" hæmolysis		
0 34–0 40% NaCl	50%	,,	(M C F.)
0 42–0.46% NaCl	"Initial"	,,	

2 Modified method of Parpart *et al*

0 30% NaCl	97–100% hæmolysis
0 35% NaCl	90–99% ,,
0 40% NaCl	50–90% ,,
0 45% NaCl	5–30% ,,
0 50% NaCl	0–5% ,,
0 55% NaCl	nil

Sedimentation rate (Wintrobe and Landsberg)

Men	Mean	3.7 mm in one hour
	Range (95%)	0–9 mm ,,
Women	Mean	9 6 mm in one hour
	Range (95%)	0–20 mm. ,,

of Dr P L Mollison

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Women	11.5 g.–16 4 g. ,,
Infants at birth (cord blood)	14.0 g.–19.0 g.* ,,
Children, 1 year	11.2 g (average) ,,
Children, 10 years	12 9 g. (average) ,,
Packed cell volume	
Men	40–54%
Women	36–47%
Infants at birth (cord blood)	44–62%*
Children, 1 year	35% (average)
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Mean corpuscular diameter (dry films)	
Adults	6.6–7.7 μ.
Reticulocytes	
Adults	0 2–2%
Infants at birth	2–6%*

* Unpublished observations

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(2)

(3)

(4)
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(7)

(8)

(9)

(10)
[illegible] , 110, 2008

(11)

(12)

(13)
J Lab clin Med, 27, 937

million red cells per c.mm. or 1 5 g. hæmoglobin may be observed. It is not so clear whether emotional activity or "random" exercise significantly raises the red cell count or hæmoglobin above the basal level observed with the subject at rest; the effects may be small enough to be submerged in the technical errors of estimation. Diurnal variation of considerable degree has, however, been claimed by various authors; for instance, McCarthy and Van [illegible] moglobin [illegible] the same [illegible]; random [illegible] t.

Physiological Variation in the Total Leucocyte Count *(6, 7, 8)*

The effect of age is indicated in the table, in general, higher levels are observed in childhood. Lymphocytes are the predominant cells from the second week of life until the fifth *to seventh year, when they give way to the polymorphs. The* count is not affected by sex. People differ considerably in leucocyte levels, but tend to maintain a relatively constant picture despite irregular oscillation during which it is claimed variation up to 50 per cent of the total count may occur The minimum count is found in the morning with the subject at rest, the maximum in the afternoon "Random activity" may slightly raise the count, severe exercise may cause rises up to 30,000 per c.mm. chiefly due, it is thought, to liberation into the blood stream of polymorphs formerly sequestrated in shut-down capillaries

A factor contributing to the apparent normal variability in leucocyte counts is the use of "capillary" blood. Leucocytes seem to become sequestrated in the capillaries of the skin, particularly if the part is cold and this leads to the counts being higher in peripheral than in venous samples. Hence the very great importance of warming the part punctured and the necessity for a free flow of blood

Lymphocytes from lymphatic channels may sometimes also enter the peripheral blood during exercise Adrenalin acts in the same way as does exercise by increasing the circulatory rate, and emotion may possibly cause an increase in the same way. The effect of the ingestion of food is uncertain, a "digestive" leucocytosis still remains unproven.

little pressure will cause the appearance of Newton's refraction rings. The cover-slip should be of such a size that when placed correctly on the counting chamber the central ruled

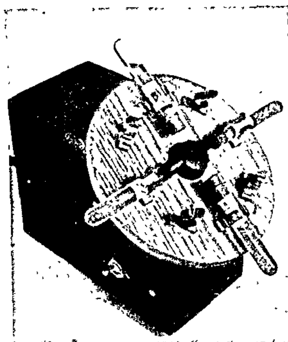


FIG 2 AN ELECTRICALLY DRIVEN MACHINE FOR MIXING SUSPENSIONS OF BLOOD CELLS (designed by Mr R E. Davis)

area or areas should lie in the centre of the rectangle to be filled with the cell suspension

The type of counting chamber used and the arrangement of the rulings is largely a matter of taste and availability The visibility of the rulings is as important as the accuracy of calibration

Count the cells using a 1/6th in. objective and a ×6 or ×10 eyepiece (the latter preferably). It is important to count as

CHAPTER 2

BASIC HÆMATOLOGICAL TECHNIQUES I

The total red cell count. The reticulocyte count. Estimation of hæmoglobin. Determination of packed cell volume (hæmatocrit value) Diagnosis of "anæmia."

THE TOTAL RED CELL COUNT

A CONSIDERABLE proportion of a technician's time is taken up in performing red cell counts. It is important that both the blood counter and the clinician who asks for the count to be made have a clear idea of the limitations of the method as usually carried out. The following technique is recommended :—

Make a 1:200 (or 1·250) dilution in formol-citrate solution by delivering by means of a "hæmoglobin" pipette 0.02 ml. *of blood into 4 (or 5) ml of diluting fluid contained in a* 80 × 10 mm tube. The blood-diluent suspension should be mixed for at least two minutes by tilting after corking through an angle of about 120° combined with rotation, thus allowing the air bubble to mix the suspension ; alternatively the cell suspension may be mixed in a mechanical mixer (Fig. 2). The counting chamber, with its cover slip already in position, should be filled without delay. This is simply accomplished with the aid of a Pasteur pipette. Care should be taken that the counting chamber is filled in one action and that no fluid flows into the surrounding moat. The chamber should be left undisturbed for at least three minutes for the cells to settle, but not much longer than this, for drying at the edges of the preparation may initiate disturbing currents which cause movements of the cells after they have settled. It is important that the cover-slip should be of a special thick glass and perfectly flat, so that when laid on the counting chamber a

label and handle. Mixing is difficult to accomplish and filling the counting chamber with the pipette so that the exact amount of fluid is delivered, is an art difficult to master. 0.02 ml pipettes of "hæmoglobin type" are relatively inexpensive and easy to calibrate. By the use of 4 ml. of diluting fluid in a corked tube, a suspension easy to label and handle is obtained, and a perfect filling of the counting chamber readily accomplished if a fine Pasteur pipette is employed.

The accuracy of 0.02 ml pipettes may be checked, after carefully cleaning them, by filling them to the mark with clean mercury, expelling the mercury and weighing it 0 05 ml of mercury weighs 680 mg., 0 02 ml. of mercury weighs 272 mg

Normal Range

Men	.	4.5–6 5 millions per c.mm.	
Women	.	3.9–5 6	,, ,,
Children.	Birth (cord blood)	4.0–5 6	,, ,,
	1 year . . .	4 5 (average)	,, ,,
	10 years	4 7 (average)	,, ,,

THE ERROR OF THE RED CELL COUNT

An accurate red cell count is difficult to accomplish. The errors associated with the count are of two main kinds; those due to inaccurate apparatus or indifferent technique (technical errors) and that due to the distribution of the suspension of red cells in the counting chamber [the inherent error or the "field error" of Magath, Berkson and Hurn (1)] The former errors can be minimized by a careful technique, the latter error can only be overcome by counting large numbers of cells.

The distribution of red cells in the counting chamber is of an irregular (random) pattern, even in a perfectly mixed sample. The pattern of distribution, however, conforms to a definite type Experiment has shown that variation between the numbers of red cells which settle in areas similar in size conforms closely* to a mathematical distribution (*Poisson*

* Actual determination of the distribution of red cells has shown a systematic but relatively unimportant deviation from the theoretical expected Poisson distribution In practice $\sigma = 0.92\sqrt{m}$, instead of $\sqrt{m}$ (1, 4)

many cells as possible, for the accuracy of the count is increased thereby (see below); 500 cells should be considered the absolute minimum. Using a Burker chamber, count the cells in three (or six) of the $3 \times 1/20$ mm. narrow rectangles, including the cells which touch the top and right hand margins. With a Neubauer chamber, count the cells in four or eight horizontal or vertical rectangles of $1 \times 1/20$ mm. (80 or 160 small squares) or in 5 or 10 groups of 16 small squares; include the cells which touch the top and right hand margins and omit from the count those which touch the lower and left hand margins of the squares.

Calculation (1:200 dilution). Burker chamber. No. of cells in $3 \times (3 \times 1/20)$ mm. narrow rectangles, 1/10 mm. in depth (9/200 c.mm. in volume) $= N$

∴ Red cell count in millions per c.mm.

$$= N \times \frac{200}{9} \times 200 \text{ (dilution)}$$
$$= N \times 4440.$$

Neubauer chamber No. of cells in $4 \times (1 \times 1/20)$ mm. rectangles, 1/10 mm. in depth (4/200 c.mm. in volume) $= N$

$$\text{Red cell count} = N \times \frac{200}{4} \times 200 \text{ (dilution)}$$
$$= N \times 10{,}000 \text{ in millions per c.mm.}$$

Red Cell Diluting Fluid. 1 per cent formalin in 3 per cent sodium citrate. This diluting fluid is very satisfactory. It is simple to make up, keeps well, does not need to be sterilized, does not cause agglutination of the corpuscles and well maintains their discoidal form The cells are well preserved and counts may be performed several hours after the dilution has been set up.

Notes on Apparatus

Bulb diluting pipettes are not recommended; they are expensive, difficult to calibrate and easily broken; they use unnecessarily small volumes of blood and are difficult to

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series) and that the standard error (σ) of the distribution of the number of cells in areas of equal size is given by $\sqrt{m}$, where m is the mean number of cells in the areas concerned. That is to say that if the red cell suspension was such that the mean number of cells in an area (say 80 small squares in a counting chamber) was 100, then if it were possible to count the number of cells in each of 100 similar areas, in 95 areas the number of cells encountered would probably range between 80 and 120 $[100 \pm 2\sigma(2 \times \sqrt{100}) = 100 \pm 20]$; in the remaining five areas the counts would be outside the 80–120 range.

Clearly this random distribution has a very important bearing on the accuracy of blood counts, for no amount of mixing will minimize the inherent variation in numbers between area and area.

If this simple calculation is applied to a concrete example, some idea of the effect of this random distribution can be obtained. Let us pretend that a blood with a true count of 5,000,000 red cells per c mm is counted in a 1:200 dilution. The mean number of cells (m)
be 500.
totals lil
squares
practice Assuming that the standard error of the number of cells in an area of 80 small squares is $\sqrt{500} = 22$ (approx.), then in 19 out of 20 areas (95 per cent) the number of cells encountered would range between $500 \pm 2 \times 22 = 456$ to 544 These figures represent a blood count ranging between 4.56 to 5 44 millions per c mm with chance determining the actual number of cells present within the area selected for the count This very great range in possible results dependent upon the random distribution of the red cells is, however, not quite so serious as it might appear to be at first sight, for whilst the number of cells encountered in one out of every 20 areas will be outside the range given above, in two-thirds of the areas the number of cells present will fall between the mean and $\pm$ its standard error ($\pm\sigma$), i e between the range 478 and 522.

The ratio $\frac{\sigma}{m}$ is the coefficient of variation (v) and this expressed as a percentage gives a convenient way of expressing the inherent error of blood counts. In the example given above $V = \frac{\sigma}{m} = \frac{22}{500} = 4.4$ per cent, and the range $\pm 2V$ involves

an error of $\pm$8.8 per cent or $\pm$440,000 cells out of a total of 5,000,000.

The *inherent* error in blood counting can be reduced in only one way; by counting more cells. This may be done in one of two ways; by counting more cells in one preparation or by making successive counts. The following calculations (Table II) demonstrate that the coefficient of variation (V) of the count varies in theory in proportion to the square root of the number of cells counted, i.e. if four times the number of cells are counted the coefficient of variation is halved. For example, if the imaginary (ideal) figures given in Table II are studied, it will be seen that 19 out of 20 counts of the cells in 80 small squares will lie within the wide range of 5.12–6.08 millions per c mm.; if the cells in 320 small squares are, however, counted the range will be considerably narrower, 5.36–5.84 millions per c.mm. (*cf.* Table III).

Table II

No of small squares counted	No of cells counted (m)	Standard error ($\sqrt{m}$)	Range (95 %) ($m \pm 2\sigma$)	Calculated blood count range ($m \pm 2\sigma$) Mill per c mm	Coefficient of variation (V) %
80	560	24	512–608	5 12–6 08	4.3
160	1120	33	1054–1186	5 27–5 93	2 9
320	2240	47	2146–2334	5 36–5 84	2.1
640	4480	67	4346–4614	5 43–5 76	1 5

Table showing how the inherent error of red cell counting may be reduced by counting larger numbers of red cells

The method of making serial counts and taking the mean has been widely (perhaps unconsciously) used as a means of reducing the error of the red cell count. If a sufficient series is done, the truth is likely to reveal itself, not only will the inherent error be reduced by the counting of a large number of cells, but there is a chance that errors in technique will in time cancel each other out The following daily counts performed on a normal subject illustrate the sort of answer which will be obtained (Table III).

The faults in technique which contribute to the *technical* error of red cell counting are many They include bad

Table III

Day	1	2	3	4	5	6	7.	8.	9	10	11.	12	Mean	σ	V.
Total red cells counted in 80 small squares.	548	555	562	574	550	598	543	498	614	571	558	551	560	28	5.0%
Corresponding red cell count Mill per c mm	5 48	5 55	5 62	5 74	5.50	5 98	5.43	4 98	6.14	5.71	5.58	5 51	5.60		
Total red cells counted in 320 small squares	2177	2198	2205	2267	2289	2408	2205	2079	2301	2253	2147	2164	2224	82	3.7%
Corresponding red cell count Mill per c mm	5 44	5 50	5 51	5 67	5 72	6.02	5.43	5 21	5 75	5 63	5.37	5 41	5 56		

The results of daily blood counts performed on a healthy adult. Freely-flowing peripheral blood was obtained by ear prick and the same pipette and counting chamber used throughout. The cells were counted by the same (experienced) observer at his usual speed using the 1/6in objective and ×6 eyepieces. The mean figures for the counts based on 80 or 320 small squares are almost identical. The coefficient of variation has, however, been reduced, but not halved, by counting the larger number of cells. The overall observed range of 5 21–6 02 million per c mm. is produced by the summation of the inherent and technical errors of the counts as well as by a possible real day to day variation. These figures give an idea of the normal order of variation (±7 5 per cent approximately) which must be borne in mind when assessing the significance of counts based on the counting of approximately 2,000 cells.

sampling of the blood due to an inadequate flow from a skin puncture, to prolonged use of a tourniquet or to inefficient mixture of venous blood which has sedimented after collection; inaccurate pipetting and the use of badly calibrated pipettes or counting chambers; inadequate mixing of the red cell suspension; a bad filling of the counting chamber and careless counting of cells within the chamber.

were carrying out some serial blood counts using the same suspensions. The figures of one of them were, nevertheless, noted to be less than those of the other, the difference in six successive counts was in the same direction, the counts of observer B averaging 6 per cent less than those of observer A The two observers then counted in succession the cells in the same 320 small squares of a single filling of a counting chamber, observer B counted 1976 cells and observer A 2086, a difference of about 5 per cent The actual number of cells in 4 outer 1 mm $\times 1/20$ mm. rectangles (80 small squares) of the Neubauer chamber where the discrepancies seemed the greatest was then determined, using $\times 10$ eyepieces and laboriously and slowly counting cell after cell —the two observers were then in agreement

The results are illustrated by the following figures —

	Observer A	*Observer B*	*"True" value*
	135	107	134
	120	108	121
	156	128	143
	124	119	142
Totals (80 small squares)	535	462	540

The experiment was repeated the following day, with much better results (there was no attempt to count 2000 cells)!

	Observer A	*Observer B*	*"True" value*
	128	137	124
	145	136	145
	125	126	126
	140	150	143
Totals (80 small squares)	538	549	538

of
adi
ter
it is difficult to maintain the necessary degree of concentration

is a high one. In fact, with counts of 5 million red cells per c.mm. and over, it is probably wise to increase the dilution of the blood to 1:400 With the Neubauer chamber it is easiest to count accurately within the centre 1 mm. square area of small squares.

The summation effect of the technical and inherent components of the errors of red cell counting as performed in a clinical laboratory has been considered by Magath, Berkson and Hurn (1, 2). They calculated the standard deviation embracing errors of sampling, pipetting and in the calibration of the counting chamber as well as the inherent error due to the random distribution of the red cells to be 385,000 per c.mm. in a 5.0 million red cell count counted in 80 small squares at a dilution of 1:200, with a coefficient of variation of 7 7 per cent (the coefficient of variation due to the inherent error is 4 4 per cent) Berg (3) in a detailed exposition accepts the calculations of Magath, Berkson and Hurn (1, 2) and gives the standard deviation as 390,000 cells and coefficient of variation as 7.8 per cent under "normal conditions of careful counting" These figures mean that in repeated estimations on blood containing 5 0 million red cells per c mm two-thirds of carefully performed counts would range between 4 61 and 5 39 millions per c mm ($\pm\sigma$) and that 95 per cent of the observed figures would lie between 4 22 and 5 78 millions per c.mm ($\pm 2\sigma$), an enormous range! The coefficient of variation is increased in lower counts if the cells in 80 small squares only are *counted. At the 3 millions level it is 8 3 per cent and at 2 millions* per c.mm 9 0 per cent

Clearly the errors of blood counting are very considerable. That due to the random distribution of the cells in the counting chamber can be reduced by counting the cells in a larger area, as already mentioned (see Table II), but in ordinary laboratory practice there is rarely time to count carefully the cells in more than 160 small squares, about 1000 cells in a normal count. The practice of making counts in duplicate is a good one, but does not necessarily increase accuracy it is always possible that the second count will be

further from the truth than the first, due to the random distribution of cells, and the error thereby increased.

As Ponder (4a) points out, the figures of Magath, Berkson and Hurn (1, 2) and Berg (3) illustrate the errors of red cell counting at their worst, and show the futility of basing results on counting the cells in 80 small squares only (usually 500 cells or less). Ponder (4a) correctly points out that if a sufficient number of cells is carefully counted and standardized pipettes and chambers are used, the error is small (not more than ±3 per cent), which is not worse than that of many other laboratory estimations! In the hands of a good, unhurried technician, using good apparatus, the technical errors of red cell counting can be almost abolished

A recent additional study of the errors of hæmatological methods has been made by Biggs and MacMillan (5, 6). Their results agree substantially with those of Berkson, Magath and Hurn (2) and with those of Berg (3). They point out the importance of personal bias in the performance of counts, and that knowledge of what the result should be leads to inaccuracy, e g. five counts made on five samples of blood will more closely correspond if the blood counter knows that the five samples are part of a single large sample. In performing a count on a single filling of a counting chamber, there is often an unconscious tendency to minimize the differences in the cell counts from successive areas, thus the number of cells in the first area counted may determine to some extent the final result Another cause of error is due to selection of counting areas. There is a tendency to select areas whose cell content seems to fit in with a preconceived idea as to what the result should be Undoubtedly, the best red cell counts are made by an unhurried, honest, methodical worker who has no knowledge of what the result should be He should always count the cells in the same areas in the counting chamber.

Another source of error in blood counts has been demonstrated by Hynes (7), this is an uneven distribution of cells within the counting chamber, depending upon the momentum given to the cells as the chamber is filled His results were obtained in the course of leucocyte counts, but probably the same principles apply to the distribution of red cells in the counting chamber He

recommends, therefore, that the centre of the cover-slip should exactly overlie the ruled area where the counts are performed.

Attempts have been made to count red cells and measure their volume by turbimetric (opacimetric) methods. The difficulty here is that variation in corpuscular size and shape and hæmoglobin content and probably unknown factors have an important effect which can only be partially compensated for, even if the hæmoglobin concentration and corpuscular size and variation are accurately known beforehand (8) It has been calculated that there is about a 1:10 chance that turbimetric methods will show a difference of more than $\pm$ 500,000 per c.mm. between red cell counts (of true value 5,000,000 per c.mm.) where in fact no differences exist (4b).

THE RETICULOCYTE COUNT

Reticulocytes are considered to be juvenile red cells; they contain within the cell membranes a proportion of the basophilic material, probably ribonucleoprotein, which was present in larger amounts in the cytoplasm of the nucleated precursors from which they were derived. This basophilic material has the property of reacting with certain dyes such as brilliant cresyl blue and forming a blue precipitate in the form of granules or a filamentous network. This reaction only takes place in unfixed preparations, if the blood is allowed to dry and is afterwards fixed with methyl alcohol the basophilic material appears as a diffuse basophilia when stained with basophilic dyes and the film as a whole will exhibit "polychromasia" The most immature reticulocytes are those with the largest amount of precipitable material, in the least immature only a few dots or short strands are seen. Ripening with complete loss of basophilic material probably occurs in the blood stream after delivery from the marrow and takes several days—the exact time is uncertain.

Reticulocytes have been classified by Nizet (9) according to the amount of [illegible] —*commends that they* be counted [illegible] There is certainly sometimes [illegible] cell is or is not a reticulocyte, even in the best preparations. Particularly is this true after splenectomy, for granular basophilic stippling of a different type is then encountered (persisting probably throughout

the life of the red cell and of a different significance). This post-splenectomy stippling [the granules have been referred to as "Pappenheimer bodies" (10)] also shows up when cresyl blue is used to stain for reticulocytes; the basophilic material is usually in the form of single or rarely multiple dots which stain a blacker shade of blue than does the filamentous material of the reticulocyte. Occasionally in hæmolytic anæmia after splenectomy, "Pappenheimer bodies" may be present in the majority of the corpuscles

In clinical hæmatology, the enumeration of the reticulocytes is one of the most important observations that can be made; the result expressed either as a proportion of the total red cells or in absolute numbers per c.mm. is in general a valuable indication of the activity of the bone marrow.

A Technique for the Reticulocyte Count

Many methods have been proposed; the following technique gives reliable results

The diluting fluid used is prepared as follows :—

0.4 g. brilliant cresyl blue is dissolved in 100 ml. citrate-saline solution (1 part 3 per cent sodium citrate to 4 parts 0.85 per cent sodium chloride).

Approximately 1 ml. volumes of the freshly filtered staining solution are delivered into 80 × 10 mm. tubes One to two drops of blood are added to a tube and mixed with the diluent. The tubes are corked and allowed to stand for 15–30 minutes at 37°C., then centrifuged for two minutes at about 1000 rev. per minute. The supernatant is removed by pipette leaving a volume of fluid not greater than twice the volume of the button of cells. The cells and supernatant are well mixed and thin films made on chemically clean slides

Reticulocytes are fragile cells and tend to break up when spread suspended in saline This tendency can be minimized if a drop or two of compatible plasma or serum is added to the deposit, and the cells suspended in this before films are made

The number of reticulocytes per 1000 *red cells* may be counted in the unfixed films or after fixing the vitally stained films in methyl alcohol for three minutes and then staining in 0.1 per cent aqueous methylene blue for 1–2 minutes, followed by a rapid rinse in distilled water (pH 6.8). Alternatively, the

films may be counterstained as for blood films with Leishman's stain or Jenner-Giemsa after fixation in methyl alcohol.

An area of film should be chosen for the count where the cells are unbroken and undistorted and where the staining is good. A common fault is to make the film too thinly; the cells should, however, not overlap. It is useful to have an eyepiece containing an adjustable square diaphragm within it, or, at least, a paper or cardboard diaphragm in the centre of which a small square with sides 2–4 mm. in length has been cut.

If the reticulocyte count is low (<5 per cent) and the film evenly spread, it is best to count the total number of cells in, say, 10 or 20 field areas of 20–30 cells each, work out an average, say, 25 cells, and then enumerate the reticulocytes in each of 50–200 successive fields. In this way the number of reticulocutes amongst 2000 or more cells may be rapidly counted. With higher counts it is more satisfactory to count the total reticulocytes in each of a series of successive fields until sufficient cells have been inspected (see Table IV).

Haldane (11) has shown that if amongst a population of cells a *total of abnormal* cells (*in this case the reticulocytes*) *is counted* during the course of examining a large number (N) of normal cells, the standard error can be derived from the formula $\sigma = p\sqrt{\dfrac{1-p}{m-2}}$ when p is the proportion of abnormal cells and m the number of the abnormal (minority) cells counted. Thus to get a standard error of 10 per cent the number of abnormal cells to be counted varies from 102 when the proportion (p) is small, e.g. 0.01 (1 per cent), to 72 when the proportion is high, e.g. 0.3 (30 per cent). *Appreciation of this fact allows a considerable saving of time*, e.g. in theory it is not necessary to survey more than 240 ($\frac{72}{30} \times 100$) cells to obtain a standard error of 10 per cent where 30 per cent of the cells are abnormal. Conversely where there are only 1 per cent of abnormals it may be necessary to survey as many as 10,100 ($\frac{101}{1} \times 100$) cells in order to obtain a standard error of the same dimensions. Fortunately, however, in clinical work such accuracy is unnecessary, and with low counts a standard error of 20 per cent or even more is of little clinical significance.

The figures quoted above are obtained in the following way: if

the desired standard error of the proportion of reticulocytes is 10 per cent, then $\sigma = \frac{p}{10} \therefore \frac{p}{10} = p\sqrt{\frac{1-p}{m-2}}$, or $\frac{1}{10} = \sqrt{\frac{1-p}{m-2}}$, or $\frac{1}{100} = \frac{1-p}{m-2}$ whence $m-2=100(1-p)$. When $p=0.01$, $m=101$, when $p=0.05$, $m=97$; when $p=0.3$, $m=72$, and so on. For practical [illegible]

that must be inspected to give approximately the same degree of accuracy of the reticulocyte count at different reticulocyte percentages. The figures have been calculated from the above formulæ.

Table IV

Desired degree of accuracy (standard error) (σ)	Percentage of Reticulocytes					
	1%	2%	5%	10%	25%	50%
20%	2525	1225	475	225	100	25
10%	10,100	4900	1900	900	400	100
5%	40,400	19,600	7600	3000	1600	400

Table [illegible]

between $P \pm 2 \times \frac{20P}{100}$, e g if P was 5 per cent, then $P \pm 2\sigma = 5 \pm 2$ per cent. At least 475 cells would have to be counted to obtain this degree of accuracy.

In practice, the error of reticulocyte counting is even greater than is inherent in a binomial distribution (5). The other factors which contribute to its inaccuracy are technical ones, the success or otherwise of the staining, the spreading of the film, the visual acuity and patience of the observer, all being of the highest importance.

ESTIMATION OF HÆMOGLOBIN

The large number of methods which have been suggested for the estimation of hæmoglobin is evidence of the real difficulty of this most important estimation. This difficulty

has centred around the preparation, permanence and calibration of the standard used for comparison with the blood under test. Although it is not possible to prepare a permanent standard of oxyhæmoglobin, solutions of certain derivatives such as alkaline hæmatin and cyanhæmatin keep relatively well and have been quite widely used in techniques in which the hæmoglobin sample to be tested is converted into the same derivative. The use of acid or carbon monoxide to convert the hæmoglobin to acid hæmatin or carboxyhæmoglobin has in the past been very popular, although as generally employed neither of these procedures has much to commend it.

In the following section five procedures will be discussed, and their merits and disadvantages indicated. Haldane's (carboxyhæmoglobin) method and the Sahli's (acid hæmatin) method are included for historical reasons rather than because they are recommended. The other methods described are the alkaline hæmatin and cyanhæmatin, requiring the use of a photoelectric colorimeter, and an oxyhæmoglobin method employing a visual reading photometer [M.R.C. grey-wedge photometer (12)]. There is little to choose between these three methods in respect of their accuracy; the alkaline hæmatin procedure is possibly the least accurate of the three.

The hæmoglobin content of a solution may be estimated for standard or reference purposes by one or all of several methods; by measurement of its colour, its power of combining with oxygen or carbon monoxide, or by its iron content. The clinical methods now to be described are all colour matching methods, which measure at the same time with varying efficiency any proportion of inert pigments (methæmoglobin or sulphæmoglobin) which may be present. Ideally perhaps, as a functional estimation of hæmoglobin, measurement of oxygen capacity should be carried out, but this is hardly practicable in clinical practice. It gives results at least 2 per cent lower than the other methods (16)—a small proportion of inert pigment is probably always present. Estimation of iron content is an accurate technique but again impracticable for clinical purposes. Estimations based on iron content are generally taken as authentic, but here, too, iron bound to inactive pigment is included. Iron content is converted into hæmoglobin content by assuming the following relationship, 100 g. hæmoglobin = 0.334 g. iron.

Normal Range

Men	13.5–18.0 g. per 100 ml. blood		
Women . . .	11.5–16.4 g.	,,	,,
Infancy (cord blood full term)	14.0–19.0 g.	,,	,,
1 year .	11.2 g. average	,,	,,
10 years .	12.9 g. ,,	,,	,,

Estimation of Hæmoglobin by the Carboxyhæmoglobin Method (Haldane)

Technique. 0 02 ml. of blood are washed into ammoniacal distilled water or N/150 NaOH* previously placed in the graduated tube up to the 20 mark. Coal gas is passed through the solution at a moderately brisk rate for at least two minutes by means of a glass capillary pipette. Frothing is prevented by first dipping the point of the pipette in caprylic alcohol. The carboxyhæmoglobin solution is then carefully matched with the standard The mean is taken of the readings of the last "just too dark" figure with the standard to one side of the graduated tube and the first "just too light" reading with the standard placed on the other side. The calibrations on the tube give the hæmoglobin level as a percentage.

Specification of Standard 100 per cent on the Haldane colour standard as defined by the British Standards Institution corresponds with a hæmoglobin content of 14 8 g. per 100 ml. by iron analysis and an oxygen capacity of 19.8 ml. per 100 ml (14, 15).

The Haldane method has been popular in England. Its disadvantages are that the standard (diluted blood saturated with carbon monoxide) has an uncertain life, a supply of coal gas is essential, certain types of blood pigment (methæmoglobin and sulphæmoglobin) are resistant to gassing and their presence and that of excess bilirubin may seriously prevent colour matching, and that it is a continuous dilution method Despite these weaknesses, the Haldane method in careful hands is quick and reasonably accurate. Its possibilities have been critically assessed by Macfarlane (13) Excluding errors in the apparatus, a test of 60

* The use of slightly alkaline water as a diluting fluid prevents the turbidity which often follows gassing This is probably due to volatile acids such as sulphur dioxide in the gas causing a precipitation of globulin.

observers showed a range of personal variability ($\pm 2 \times$ the coefficient of variation) of 2.0 to 13.9 per cent; the average was 4 8 per cent. In its favour are the facts that a B S I. colour standard has been defined against which standards may be compared, and that B S.I specifications exist for pipettes and graduated tubes. It can be used fairly accurately in artificial light.*

The Acid Hæmatin Method

Technique. The graduated tube is filled to the 20 mark with N/10 HCl. 0.02 ml. of blood are washed into it. After five (or ten) minutes the brown solution is diluted with N/10 HCl until the colour of the solution matches that of the standard. The level of hæmoglobin is directly read as a percentage or in grams from the calibrations on the graduated tube. The best simple apparatus has two standards between which the graduated tube is placed. As the standards are usually of plane glass, a tube square or rectangular in section is to be preferred.

Specification of Standard. This varies with the maker and may be given as anything between 13 and 17 g. per 100 ml. blood. It should always be checked by other methods.

Although a number of hæmoglobinometers using this principle have been on the market, this popular method has serious *disadvantages* The *standards, usually of brown glass, vary in their* supposed hæmoglobin equivalent and may fade, they usually need recalibration by comparison with other methods. The brown colour of acid hæmatin takes time to develop to its full intensity depending upon the temperature and protein and lipoid content; and fœtal hæmoglobin is relatively resistant to the acid treatment Acid hæmatin is, moreover, present in colloidal form in solution, and this may result in a visibly turbid solution unless the blood is quickly and thoroughly mixed with the acid. However, the apparatus required may be as simple as that of the Haldane method, and no coal gas is needed Hence, it perhaps still has a

The development of colour

definite time At room

the total colour develops

in 1 minute; in 5 minutes, 88 per cent After 2 hours the colour development appears maximal (17 18). In practice, the standards are usually calibrated for matching after 5 to 10 minutes.

The Alkaline Hæmatin Method

The standard method (19) will be described first, and then two slightly modified techniques.

Technique. 0.05 ml. blood is added to 4.95 ml. N/10 NaOH, heated in a boiling water bath for five minutes, and then when cool matched against the alkaline hæmatin standard.

Preparation of Standard. 37 5 mg. pure hæmin* are dissolved in 1 litre of N/10 NaOH. This solution is equal in colour to a 1 in 100 dilution of blood containing 7 4 g. hæmoglobin per 100 ml. (50 per cent hæmoglobin)

This method has several advantages over the acid hæmatin method. By the substitution of alkali for acid, total pigment is estimated; carboxyhæmoglobin, methæmoglobin and sulphæmoglobin give the same proportion of colour as does oxyhæmoglobin, and a true solution is obtained. The plasma proteins and lipoids have little effect on the development of colour, but may cause turbidity unless blood and alkali are quickly and thoroughly mixed The standard alkaline hæmatin solution can be prepared from crystalline hæmin of known iron content and should keep its colour for at least three months A disadvantage of the method is that certain forms of hæmoglobin are resistant to alkali denaturation, in particular that in fœtal or neonatal blood This is overcome by heating the solution in a boiling water bath for 5 minutes Matching can be carried out in a Duboscq colorimeter or in a photoelectric colorimeter A green filter is employed, bright spectrum yellow green Ilford No 625, is suitable

A Modified Alkaline Hæmatin Method—the Acid-alkali Method

A disadvantage of the alkaline hæmatin method as previously described is that heating of the solution of hæmoglobin is required in order to complete the denaturation by alkali This procedure can be omitted if the blood is collected first into acid, and, after standing for 20–30 minutes, sufficient alkali is added to neutralize the acid and convert the acid hæmatin into alkaline hæmatin

Technique 0 05 ml blood is washed into 4.0 ml. N/10 HCl and immediately well mixed with it. After standing

* Pure crystalline hæmin contains 8 57 per cent Fe Less pure samples of hæmin should be used proportionately to their Fe content

for 20–30 minutes, 0.95 ml. of N. NaOH is added, and the tube inverted several times. The test sample can then be matched with the standard in a photoelectric colorimeter after standing for not less than two minutes. As in the previous method a green filter is employed.

Estimation of Hæmoglobin by the Alkaline Hæmatin Method using Gibson and Harrison's Artificial Standard

Recently, an apparently permanent standard for use with the alkaline hæmatin procedure has been described (Gibson and Harrison (20)) This is a mixture of chromium potassium sulphate and cobaltous sulphate in aqueous solution, and may be made up in almost any laboratory. Pure and anhydrous chemicals are required. Details are given in Gibson and Harrison's (20) paper (see also Appendix I). The standard is equivalent to 16 0 g hæmoglobin per 100 ml. (108 per cent).

Technique. 0 05 ml. blood is washed into and well mixed with 4.95 ml. N/10 NaOH The test sample (or samples) are then heated along with a sample of the standard in a boiling water bath for four minutes, and then cooled quickly in cold water. Matching in a photoelectric colorimeter using a green filter should be performed within 30 minutes.

It is essential to heat the standard as described. Only after heating, which alters the ionization of the salts it contains, does its power to absorb green light approximate closely to that of alkaline hæmatin A fresh sample of standard should be heated on each occasion and then discarded.

The Cyanhæmatin Method

Technique 0 05 ml blood is washed into 4 95 ml. N/10 HCl. The mixture is inverted several times and then left for at least 30 minutes so that the colour may develop. 5 ml of 2 per cent sodium cyanide solution are then added After mixing by corking and inversion the solution may be matched in a colorimeter. It is convenient to use a standard equivalent to 50 per cent hæmoglobin or 7.4 g per 100 ml

Preparation of Standard 14 5 mg hæmin are dissolved in 1 litre 1 per cent sodium cyanide The colour obtained is

equivalent to a 1:200 dilution of blood containing 7.4 g. Hb. per 100 ml. treated as above.

This technique (21) has several advantages. All forms of hæmoglobin are estimated; the standard gives a colour almost identical with the clear solution obtained by treatment of blood with hydrochloric acid followed by sodium cyanide. The standard is almost permanent, but should be checked at least every two months. Matching is best performed with a photoelectric colorimeter using a green filter This method is probably as accurate as any of the simple procedures (22), but the use of sodium cyanide is a disadvantage.

An Oxyhæmoglobin Method employing a Grey-Wedge Photometer and Green Filter

Technique. 0.02 ml. blood is washed into 4 ml. N/150 NH_4OH. After mixing by inverting several times, the solution of oxyhæmoglobin is ready for matching in the photometer. The density of light transmitted by the test solution is matched in an adjacent half field against the light transmitted by the rotating grey wedge. A green filter (bright spectrum yellow green, Ilford, No. 625) is employed. The hæmoglobin content is read off as a percentage (Haldane scale). 100 per cent = 14 8 g. Hb

With this instrument, the M.R.C. grey-wedge photometer [King *et al.* (12)], hæmoglobin is estimated as oxyhæmoglobin, and its power of absorbing green light is compared with a carefully calibrated neutral grey wedge. The instrument is portable and may be used with daylight or artificial light. In careful hands it gives results comparable with the best clinical methods (23). No delay in reading is necessary, and no complicated manipulations are required.

A possible cause of error is the presence of dirt on the walls of the glass cells or on the glass surfaces anywhere between the light source and the eyepiece, and which is affecting the two light fields unequally If the apparatus appears to be giving anomalous results, the first thing to do is to clean the glass surfaces thoroughly, taking it partially to pieces if necessary.

DETERMINATION OF PACKED CELL VOLUME (HÆMATOCRIT VALUE)

CALCULATION OF "ABSOLUTE VALUES" FOR MEAN CELL VOLUME, HÆMOGLOBIN AND HÆMOGLOBIN CONCENTRATION

Wintrobe's hæmatocrit tubes are now in daily use in most clinical laboratories Not only is an estimation of packed cell volume (P.C.V.) valuable as a screening test for the detection of anæmia, but, in conjunction with an estimation of hæmoglobin and an accurate red cell count, it enables figures for mean cell hæmoglobin concentration and mean cell volume to be calculated, information which is often of great value in diagnosing the type of anæmia which may be present.

The terms mean cell hæmoglobin concentration, mean cell volume and mean cell hæmoglobin are often referred to as "absolute values." Their calculation and use has rendered obsolete the various indices, such as the "colour index," derived from the comparison of observed data with figures arbitrarily assumed to be "normal."

Method of Estimation of Packed Cell Volume

Wintrobe's tubes, calibrated to 100 mm., are usually employed for the determination of the packed cell volume after centrifuging. These have a capacity of about 1 ml. Smaller uncalibrated tubes made out of barometer tubing can be used if less blood is available.

Venous blood collected with minimal constriction is rendered incoagulable with dry ammonium oxalate and potassium oxalate mixture, 2 mg per ml., or by heparin, 0.1–0.2 mg. per ml Potassium oxalate alone causes some shrinkage of the red cells and should not be used The blood should be very carefully mixed by repeated inversion until bright red, and the hæmatocrit tube then filled at once from the bottom upwards to the 100 mm. mark by means of a glass capillary pipette The tube is centrifuged at a speed of 3000 r.p.m. for 30 minutes. The height of the column of the red cells is taken as the packed cell volume (the volume occupied by the red cells expressed as a percentage). Above

the red cells and not included in the figure for the packed cell volume may be seen a greyish red layer of leucocytes and a thin creamy layer of platelets.

Normal Ranges for Packed Cell Volume (hæmatocrit value). Men, 40 to 54 per cent. Women, 36 to 47 per cent.

Calculation of Mean Cell Volume (M.C.V.). If the packed cell volume and the number of red cells per c.mm. are known, the mean cell volume can be calculated.

e.g. if packed cell volume is 45 per cent, in 1 c.mm. of blood there are 0.45 c.mm. cells.

∴ if there are 5,000,000 red cells per c mm , they occupy a volume of 0.45 c.mm.

$$\therefore \text{ volume of 1 cell (m.c.v.)} = \frac{0.45\,\text{c.mm.}}{5{,}000{,}000} = \frac{0.45\times10^9}{5\times10^6}\,\text{c}\mu = \frac{0.45\times10^3}{5}$$

$$=90\ \text{c.}\mu.$$

In practice, divide the packed cell volume as a percentage, by the red cell count in millions per c.mm and multiply by 10. Express the answer in c.μ.

$$\text{i e. } (45\div5)\times10=90\ \text{c }\mu.$$

Calculation of Mean Cell Hæmoglobin (M.C.H.). This can be calculated if the hæmoglobin and red cell count are known.

e g. if there are 15 g. Hb. per 100 ml. blood, there is

$$\frac{15}{100\times1000}\text{ g. Hb. per 1 c.mm. blood}$$

∴ if there are 5,000,000 red cells per c.mm , the mean cell hæmoglobin is $\frac{15}{100\times1000\times5{,}000{,}000}$ g. per cell $=\frac{3}{10^{11}}$ g.

$=30$ micromicrograms ($\mu\mu$g.).

Calculation of Mean Cell Hæmoglobin Concentration (M.C.H. Conc.). This can be calculated if the hæmoglobin and packed cell volume are known.

e.g. if there are 15 g. Hb. per 100 ml. blood, of packed cell volume 45 per cent, the hæmoglobin concentration is 15 – 45 g. per cent = 33.3 per cent.

Range of "Absolute Values." *Normal* (24, 25).
Mean cell volume (M.C.V.) 76 to 96 c.μ.
Mean cell hæmoglobin (M.C.H.) 27 to 32 μμg.
Mean cell hæmoglobin concentration (M.C.H. Conc.) 32 to 36 per cent.

In Disease.

In the megalocytic anæmias,
M.C.V. increased up to about 150 c.μ. (rarely higher).
M.C.H. increased up to about 50 μμg. (rarely higher).
M.C.H. Conc normal or diminished

In microcytic hypochromic anæmias,
M.C.V. diminished to 50 c.μ. (rarely lower)
M.C.H. diminished to 15 μμg. (rarely lower).
M.C.H. Conc. diminished to 22 per cent (rarely lower).

The Accuracy of the Hæmatocrit Method. Duplicate samples centrifuged at the same time will agree within 1–2 per cent. It is this reproducibility which makes the estimation of packed cell volume so valuable. It is worth while to ensure that the blood is well aerated before filling the hæmatocrit tube, as the packed cell volume of venous blood is appreciably (0.5–1 per cent) greater than that of aerated blood which has lost carbon dioxide and taken up oxygen. It is not clear whether a speed of 3,000 r.p.m for thirty minutes is the ideal speed of centrifuging, a constant volume can be obtained thereby, but "constant volumes" will be attained at speeds both faster and slower than this—the faster the speed the smaller the volume (26). 3000 r.p m is, however, within the capacity of most laboratory centrifuges and the results obtained are generally accepted.

An additional factor of importance which in association with the speed of rotation determines the centrifugal force applied to the

blood, is the radius of the circle of rotation The distance from the spindle of the centrifuge to the bottom of the hæmactocrit tube should be standardized at 15 cm. [see Reeve (27)]

A danger attached to the calculation of "absolute values" is that the observer may delude himself into a false sense of their accuracy, particularly when the results are expressed to one place of decimals, as is sometimes quite unjustifiably done! The significance and accuracy of "absolute values" naturally depends upon the accuracy of the estimations from which they have been derived. As has already been mentioned, the error of the hæmatocrit method is a small one, not more than ±1 per cent, and the same is more or less true of hæmoglobin estimation, which in the very best hands has a minimum error of about ±2 per cent. Thus it is possible to obtain a reasonably good estimate of hæmoglobin concentration. Unfortunately, the same cannot be said of the calculation of mean cell volume or mean cell hæmoglobin These estimations depend for their accuracy largely on the accuracy of the red cell count. In this case, although it is possible to reduce the error of the count to small proportions, even to as little as ±2 per cent, this can only be done by counting thousands of cells, and this is rarely carried out in practice. If the count is done in a "routine" manner and perhaps not more than 500 cells counted, the potential error is so large that any calculation of mean cell volume is almost worthless. An example will make this clear The error of a red cell count comprising a count of 500 cells in 80 small squares cannot be less than ±8 8 per cent (see p 16). Thus blood of real red cell count 5 0 millions per c mm might appear to contain anything between 4 56 to 5 44 (±2σ limits) million red cells per c.mm. If the packed cell volume of the same blood was 47 per cent, the mean cell volume would appear to be within the range 103 to 86 μ with a 1 in 20 chance of being outside the range! Hence the above blood might or might not seem to be abnormal, depending on chance selection of the counting areas In fact, repeated observations on the same blood so as to give an average figure or a single count based on several thousand cells are necessary to determine without question slight deviations from the normal.

DIAGNOSIS OF ANÆMIA: THE USE OF THE RED CELL COUNT

In the foregoing chapter have been described certain of the basic techniques of hæmatology—including red cell counting, hæmoglobin estimation, estimation of packed cell volume and the calculation of "absolute values." The purpose of this section is to consider briefly how the above techniques may be best applied to the diagnosis of the abnormal.

It should be unnecessary by now to emphasize that a reliable estimate of a red cell count cannot be obtained without taking much time and trouble, and that the larger the number of cells counted the more accurate the result will be. The logical conclusion to be drawn from this is to undertake as few red cell counts as possible, and to do them properly or not at all.

Clinical hæmatology can be divided into two main categories, the diagnosis of the presence or absence of a blood abnormality, and the investigation of this abnormality after it has been detected For the first purpose a red cell count is generally unnecessary; it is sufficient to estimate the hæmoglobin content and/or packed cell volume and inspect a stained film of the blood. Nothing is gained by the performance of a perfunctory red cell count Once the presence of "anæmia" or the converse—an unusually high hæmoglobin, has been established, then steps can be taken to investigate the condition Venous blood should be obtained and absolute values calculated. At least 1000 red cells should be counted

No system should be adhered to too rigidly, and it is fully admitted that there are occasions when reliance on an estimate of hæmoglobin or packed cell volume alone will mislead the observer In mild pernicious anæmia, for instance, only an accurate red cell count can show whether the patient is being adequately treated, e g. the hæmoglobin and packed cell volume may be within the normal range although the red cell count is 4 0 millions or less Even in these cases, however, a red cell count badly performed may be worse than useless, and more information can be gained by a careful inspection of the film.

It is no exaggeration to say that about three-quarters, at least, of the red cell counts requested by some clinicians are unnecessary ; if performed, they do not make the diagnosis any clearer, nor do they help the patient. Not only are "full" or "complete" blood counts asked for when the patient's blood is entirely normal, but often red cell counts are requested when it is perfectly clear that the patient is suffering from acute hæmorrhage and a hæmoglobin estimation is all that is necessary. A perfunctory red cell count sometimes even confuses the issue by revealing a high "colour index." By eliminating redundant, useless and of necessity inaccurate red cell counting, time can be gained for carrying out counts on those patients with blood disorders in whom knowledge of the red cell count is necessary for diagnosis or treatment or is of scientific interest

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Chapter 3

BASIC HÆMATOLOGICAL TECHNIQUES II

The preparation of blood films on slides Staining by Romanowsky dyes. Total and differential leucocyte counts. The examination of blood films.

THE PREPARATION OF BLOOD FILMS ON SLIDES

Blood films can be made on slides or cover glasses. The latter have the possible single advantage of a better distribution of leucocytes, but in every other respect slides are to be preferred Unlike cover glasses, slides are not easily broken; they may be simply labelled, and when large numbers of films are to be dealt with, slides are in every way much easier to handle.

Good films may be made in the following manner, using chemically clean slides wiped free from dust immediately before use. It is essential that the slide used as a spreader should have an absolutely smooth edge and should be narrower in breadth than the slide on which the film is to be made, so that the edges of the films may be readily examined. A small drop of blood is placed in the centre line of the slide about 1 cm. from one end. The spreading slide is then placed at an angle of 30–40° to the slide in front of the drop and then moved back to make contact with it. The drop should quickly spread out along the line of contact of the spreader with the slide The moment this occurs, the film should be spread by a rapid smooth forward movement of the spreader The drop of blood should be of such a size that the film is about 3 cm. in length The faster the film is spread the more even and thicker it is likely to be. A common mistake is to make films too thin whilst attempting to avoid the opposite. The ideal thickness is such that there is some overlap of red cells throughout much of the film's length with separation and lack of distortion towards the tail of the film. The leucocytes

should be easily recognizable throughout the length of the film. Details of their distribution in films is discussed in a later section (p. 44). Fig. 3 is a schematic drawing of a blood film made on a slide.

A Note on Labelling Blood Films. A recommended method is to write the name of the patient and the date in pencil (lead) on the film itself. It will not be removed by the staining. A paper label may be affixed to the slide later

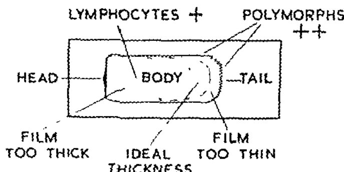


FIG 3 SCHEMATIC DRAWING OF A BLOOD FILM MADE ON A SLIDE
The film has been spread from left to right An indication is given of the way the leucocytes are distributed (see text)

THE STAINING OF BLOOD FILMS

Romanowsky stains are almost universally employed for staining blood films, and very beautiful pictures may be obtained. For the best results films should be stained as soon as they have dried in the air, and in any case should not be left for more than a few hours without fixation If films are left unfixed for a day or more, it will be found that the background of dried plasma stains a bluish tint. This is difficult to remove without spoiling the staining of the blood cells As the intensity of staining is affected by any variation in the thickness of a film, it is not easy to obtain uniform staining throughout a film's length

The remarkable property that the Romanowsky dyes have of making subtle distinctions in shade of staining and of

staining granules differentially depends upon the presence of derivatives in the stain of the interaction of methylene blue and eosin, the original Romanowsky combination. Of particular importance is "methylene blue azure" which stains the "azurophil" granules of leucocytes and the nuclei of malarial parasites a reddish colour (1). In Giemsa's stain methylene blue azure is deliberately added to the methylene blue—eosin mixture. Jenner's stain is the simplest of the Romanowsky dyes and Giemsa the most complex. Leishman's stain, which occupies an intermediate position, has been widely used in the routine staining of blood films.

All these stains are sensitive to H ion concentration, a *p*H to the alkaline side of neutrality accentuating the methylene blue component at the expense of the eosin, and vice versa. A *p*H of 6 8 is recommended for general use, but to some extent this depends on personal taste. A uniform *p*H is, however, desirable To achieve this, 2 ml. of a M/5 KH_2PO_4–NaOH buffer solution may be added to each 100 ml of the distilled water used in diluting the stains and washing the films

The mechanism by which certain components of a cell s structure stain with particular dyes and other components fail to do so, although staining with different dyes, depends upon complex differences in chemical structure and affinities. In the case of Romanowsky dyes, it is the presence of the acidic groupings of the nucleic acids and proteins of the cell nuclei and primitive cytoplasm which determines their staining by the basic dye methylene blue, and conversely it is the presence of basic groupings on the hæmoglobin molecule which results in its affinity for acidic dyes and its staining by eosin. A few further details concerning this complex subject are given in Chap 5 when Unna Pappenheim, Feulgen and sudan black staining are considered, in the case of sudan black, it is probably a question of differential solubility rather than chemical affinity or combination which determines the presence or absence of staining

STAINING METHODS

Leishman's Stain. Use air-dried films Flood the slide with Leishman's stain and leave for two minutes. Add double the volume of distilled water and leave for five to seven minutes Rock slide gently to aid mixing. Wash with a

running stream of buffered water until the film has a pinkish tinge (half to two minutes). Wipe the back of the slide clean. Shake off excess water and allow to dry in an upright position.

Jenner-Giemsa or May-Grünwald-Giemsa Combination. Use fresh air-dried films. Immerse in a jar of absolute methyl alcohol. Fix for two to five minutes. Transfer the slide to a jar containing May-Grunwald's stain diluted with two parts of buffered distilled water (*p*H 6.8) and leave for three to five minutes. Transfer the slide without washing to Giemsa's stain diluted with nine parts of buffered distilled water and leave for seven to ten minutes. Differentiate in buffered distilled water for about five minutes, controlling the result under the microscope: wipe the back of slide and allow to dry in an upright position.

This technique is designed for staining a number of films at the same time Single slides may be stained by flooding the slide with fixative or staining solution.

A relatively prolonged fixation, two to five minutes, is required for good staining; particularly is this so in staining smears of bone marrow (see p. 76).

When dry, the stained films should be covered with a cover glass. A neutral mounting medium is desirable if the slides are to be kept Gurr's medium is useful and miscible with xylol For a temporary mount, cedar wood oil may be used.

The coverglass should be sufficiently large to overlie the whole film, including both the edges and the tail If a neutral mounting medium is used the staining should be preserved for at least five years Although it is probable that stained films keep best unmounted, there are objections to this course; it is almost impossible to keep the slides free from dust and in the absence of a coverglass the observer is tempted to examine the film solely with the oil immersion objective a practice which is to be deprecated (see also p 81)

Formulæ of Phosphate Buffers which may be added to the Distilled Water used in Diluting and Differentiating Romanowsky Stains

M/5 KH_2PO_4 =50 ml.	N 5 NaOH	17.8 ml.	*p*H	6.6
50 ml.		21 0 ml.		6.7

M/5 KH_2PO_4=50 ml.	N/5 NaOH 23.7 ml.	*p*H	6.8
50 ml.	26.5 ml.		6.9
50 ml.	29.6 ml.		7.0

THE LEUCOCYTE COUNT

The Total Leucocyte Count

Make a 1:20 dilution of blood by adding 0.05 ml. blood to 0.95 ml. diluting fluid in a 75×8 mm. tube. Mix for at least two minutes by tilting and rotation. Fill the counting chamber by means of a fine Pasteur pipette and allow the cells to settle. Use the 1/6th in. objective and the ×6 eyepiece or the 2/3rd in. objective and the ×10 eyepiece. Count at least 100 cells in as many 1 square mm. areas (1/10 c.mm. in volume) as may be necessary—the ruled area in the Neubauer chamber consists of nine of these areas.

Calculation. N cells counted in 1/10 c mm ; leucocyte count per c.mm. =N×10×20 (dilution) =N×200.

Diluting Fluid. 2 per cent acetic acid coloured with brilliant green or gentian violet.

Normal Range (90 per cent of subjects)

Adults .	4000–10,000	per c.mm.
Birth .	10,000–25,000	,,
1 year .	6000–18,000	,,
Childhood (4 to 7 years) .	6000–15,000	,,
(8 to 12 years)	4500–13,500	,,

The Error of the Total Leucocyte Count

The factors causing errors in counting leucocytes are the same as in red cell counting and it is desirable therefore, to count as many cells as possible ; 100 cells is a reasonable and practical figure. The standard error (σ) of a 100 cell count is approximately $\sqrt{100}=10$ and the coefficient of variation is thus 10 per cent , 95 per cent of counts of mean value 100 would thus lie within the range $100\pm2\sigma=80-120$ Translated into actual results, this means that 95 per ... ue 5000 cells per c ... erg (2) allowing for ... echnical errors), give ... counts based on 100 cells The technical component of error can, however,

be reduced to almost negligible proportions by using calibrated apparatus and avoiding "white cell pipettes" which are often so difficult to manipulate.

Fortunately, error in leucocyte counting is not nearly so important as the error in red cell counting; even an error as high as 20 per cent does not matter much—the difference between 5000 and 6000 cells per c.mm. is of little practical significance, and perhaps less than that due to "physiological" variation. With high counts the error may be reduced by counting more cells without the expenditure of extra time. If 400 cells are counted, the coefficient of variation is reduced to 5 per cent $(\frac{\sqrt{400}}{400} \times 100)$. The observations of Hynes (2) on the unequal distribution of leucocytes in the counting chamber brought about by the momentum of filling has already been referred to. If the centre of the coverglass overlies the ruled area within which the cells are counted, this error is negligible.

The Differential Leucocyte Count

The making of blood films has already been described (p. 39), and the present discussion is concerned *solely* with the technique of performing differential leucocyte counts on blood films made on slides, the most usual method. For the purposes of a differential leucocyte count, the films must not be too thin and the tail of the film should be smooth, i.e. the films should be spread quickly. The thickness of the films should be such that there is some overlap of the red cells diminishing to separation near the tail, but it should not be so thick that the leucocytes in the body of the film are badly shrunken. If films are made too thinly, the majority of the leucocytes, perhaps 50 per cent, may be aggregated at the edges and in the tail; and even in the best films there will be an increased number of cells in these areas. Moreover, a qualitative irregularity in distribution is the rule; polymorphs and monocytes preponderate at the margins and tail, and lymphocytes in the middle of the film (Fig. 3). This, perhaps, depends upon differences in stickiness, size and specific gravity between the different classes of cells.

Various systems of performing the differential count have been advocated. The problem is to overcome the differences in distribution of the various classes of cells which are always

present to a greater or less extent. No system of counting will compensate for the gross irregularities in distribution which occur in badly made films. In films spread slowly by means of an irregularly-edged spreader, the majority of the polymorphs will be carried to the tail of the film. There they will be *seen in clumps and trails and many will be broken up and* unrecognizable. It is almost a waste of time to attempt a differential count on such a film and, if this is attempted, futile to count only the cells in the centre of the film, where lymphocytes perhaps predominate, and to neglect altogether the tail, where lie the polymorphs. If the film has been well made and many leucocytes are present in the body of the film and there is no great accumulation at the tail, the following technique of counting can then be recommended:

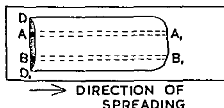


FIG. 4 SCHEMATIC DRAWING ILLUSTRATING THE LONGITUDINAL METHOD OF PERFORMING DIFFERENTIAL LEUCOCYTE COUNTS

leucocytes in one or more strips such as A–A₁, and B–B₁ must be inspected and classified

count the cells in a strip running the whole length of the film using the 1/6th or 1/12th lens, avoiding the lateral edges; count from the head of the film to the tail, and if less than 200 cells have been encountered in a single narrow strip, count one or more additional strips until at least 200 cells have been counted. Each longitudinal strip represents the blood drawn out from a small segment of the original drop of blood when it has spread out between slide and spreader (see Fig. 4); hence there is every reason to suppose that if all the cells are

be reduced to almost negligible proportions by using calibrated apparatus and avoiding "white cell pipettes" which are often so difficult to manipulate.

Fortunately, error in leucocyte counting is not nearly so important as the error in red cell counting; even an error as high as 20 per cent does not matter much—the difference between 5000 and 6000 cells per c mm is of little practical significance, and perhaps less than that due to "physiological" variation. With high counts the error may be reduced by counting more cells without the expenditure of extra time. If 400 cells are counted, the coefficient of variation is reduced to 5 per cent ($\frac{\sqrt{400}}{400} \times 100$). The observations of Hynes (2) on the unequal distribution of leucocytes in the counting chamber brought about by the momentum of filling has already been referred to. If the centre of the coverglass overlies the ruled area within which the cells are counted, this error is negligible.

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Various systems of performing the differential count have been advocated. The problem is to overcome the differences in distribution of the various classes of cells which are always

Table V (c)

No	Total cells counted.	Polymorphs.	Eosinophils	Basophils	%	Lymphocytes	Monocytes	%
11A	200	114	0	0	57	74	12	43
12A.	200	133	3	1	68.5	60	3	31 5
13A.	200	115	2	0	58.5	71	12	41 5
14A.	200	131	2	3	68	57	7	32
15A.	200	120	1	0	60.5	72	7	39.5
16A.	200	122	2	0	62	68	8	38
17A	200	112	0	4	61.5	71	6	38 5
18A	200	117	3	2	61.5	61	17	39
19A	200	125	2	0	62 5	64	9	37 5
20A	200	125	2	0	62.5	72	1	37 5
Total	2000	Mean (c)=62 2% σ=3 8				Mean=37 8%		
21A	200	141	1	0	72	42	16	28
22A	200	130	1	2	66.5	52	15	33 5
Total	400	Mean 69 3%				30 7%		

counted in such a strip, the differential totals will be representative of the true differential count This technique admittedly does not allow for the preponderance of polymorphs and monocytes at the edges of the film, but this preponderance is slight in a well made film and in practice makes little difference to the result

The above technique is very easy to carry out, with high counts (10,000–30,000 per c.mm) a short film (2–3 cm.) is desirable. In patients with very high counts (as in leukæmia)

Table V (a)

No	Total cells counted	{Polymorphs, Eosinophils, Basophils	{Lymphocytes, Monocytes
1	100	71	29
2	100	61	39
3.	100	55	45
4	100	65	35
5	100	69	31
6.	100	72	28
7.	100	69	31
8	100	71	29
9	100	73	27
10.	100	65	35
Total 1000		Mean (a) 67%	Mean 33%

Table V (b)

No	Total cells counted	Polymorphs	Eosinophils	Basophils	%	Lymphocytes	Monocytes	%
11	300	186	0	0	62	98	16	38
12	225	157	3	1	71 5	61	3	28 5
13	326	209	4	1	65 3	97	16	34 7
14	327	224	4	1	70 7	86	10	29 3
15	306	205	3	0	68	87	11	32
16	274	180	2	1	66 8	81	10	33 2
17	250	165	2	1	67 2	73	9	32 8
18	203	120	3	2	61 6	61	17	38 4
19	328	224	3	1	69 5	86	14	30 5
20	300	198	6	1	68 4	91	4	31 6
Total 2840		Mean (b) 67 1%, σ = 3 2				Mean 32 9%		
21.	417	298	2	3	72 7	87	27	27 3
22	398	288	7	2	74.1	81	22	25.9
Total 815		Mean 73 4%				Mean 26 6%		

40 per cent to 60 per cent polymorphs, if 100 cells are counted With 500 cells counted the range would be reduced to 4.4 per cent, e g. 45.6 per cent to 54.4 per cent polymorphs. With

less, and below

standard devia-

because they can only be applied to well spread films. It is waste of time to subject to statistical analysis results obtained from a badly spread film.

Normal Range of Differential Leucocyte Counts in Adults

Polymorphs . .	40–75%	2500–7500 per c.mm
Lymphocytes . . .	20–45%	1500–3500 ,, ,,
Monocytes . .	2–10%	200–800 ,, ,,
Eosinophils	1–6%	200–400* ,, ,,
Basophils . . .	1%	15–100 ,, ,,

THE DIAGNOSIS OF BLOOD DISEASES BY EXAMINATION OF BLOOD FILMS

This is largely a matter of experience; but the observer is greatly helped by a good technique. It is impossible to over-emphasize the importance of making good films of the correct thickness and of staining them well. A definite technique of examination should be followed. First, the films should be looked at under a low magnification. An idea of the type of film, good or bad, will be learnt thereby, and of the number, distribution and staining of the leucocytes, in this way also an area of even distribution where the red cells are not distorted can usually be found even in the worst films.

* The recent demonstration of a relationship between the eosinophil count and the activity of the adrenal cortex (7), has underlined the importance of

method in

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the technique has to be abandoned, and the cells counted in any well spread area where the cell types are easy to identify.

Other systems of counting, such as the "battlement" count (4), seem to be more elaborate and have no advantage. In the "battlement" count, the cells in three horizontal edge fields are first counted, then those in two vertical fields, then in two horizontal fields, and then in two vertical fields back to the edge once more, and so on—three edge fields alternate with five internal fields. This seems to weight the count too highly in respect of edge fields, for in a film of 2 cm. width there may be 50 or so fields across the width of the slide, when the $\frac{1}{6}''$ objective is employed.

In Table V are given details of an experiment which demonstrates the reliability of the longitudinal strip method of performing differential counts. The blood was from a normal subject. The results are compared with a differential performed on a "thick film" (see p. 153) of the same blood. As it is difficult to separate lymphocytes from monocytes in thick film preparations, they were classed together for the purposes of this experiment. Table V illustrates that the counts based on longitudinal strips gave approximately the same result as the counts based on the thick film. The Table also gives an idea of the variation which may be expected between the counts on different strips if 200–300 cells are counted. It also demonstrates the increased numbers of leucocytes (and increased percentage of polymorphs) present at the margins of the films, and that counts performed on the head and body of the film give significantly lower polymorph totals than are obtained when complete (head to tail) strips are counted, i.e. polymorphs are present in higher proportion at the tail.

The variation between the counts made on different strips from the same film, as recorded in Table V, illustrates that the observed differential count depends not only on artificial differences in
[illegible] "random"
[illegible] counting a
[illegible] nd practical
studies (5, 6) have shown that with a true polymorph count of 50 per cent the range ($\pm 2\sigma$) within which 95 per cent of the counts will fall, if 200 cells are counted, is of the order of $\pm$ 7 per cent, e.g. 43 per cent to 57 per cent polymorphs, and $\pm$ 10 per cent, e.g.

(6) STURGIS, C C., and BETHELL, F. H (1943). "Quantitative and qualitative variations in normal leukocytes" *Physiol. Rev*, 23, 279
(7) THORN, G. W., FORSHAM, P H, PRUNTY, F T G, and HILLS, A. G. (1948) "A test for adrenal cortical insufficiency; the response to pituitary adrenocorticotropic hormone" *J Amer med. Ass*, 137, 1005
(8) RANDOLF, T G (1949) "Differentiation and enumeration of eosinophils in the counting chamber with a glycol stain; a valuable technique in appraising ACTH dosage" *J Lab clin Med*, 34, 1696.

Having selected a suitable field, the 1/6th objective should be used next. A much better appreciation of variation in red cell size, shape and staining can be obtained with this objective than with the oil immersion lens. The latter in combination with the ×6 eyepiece should be reserved for the final examination of unusual cells and for looking at fine details such as basophilic punctation of the red cells, etc.

Although the hæmatologist should not be asked to make a diagnosis from a blood film without any knowledge of the hæmoglobin, total red cell or leucocyte counts, etc., films are often presented and a diagnosis asked for. It is here that experience helps, but this is no substitute for a good technique of examination. The diagnosis of the type of anæmia or abnormality present usually depends upon a comprehension of the whole picture which the film presents, and the red cells, leucocytes and platelets should, therefore, all be systematically examined. Two examples will illustrate this—the differential diagnosis between leukæmia and glandular fever depends not only on differences in the leucocytes, but also on the almost invariable presence of anæmia, with red cell changes and thrombocytopenia, in leukæmia; in acute hæmolytic anæmia, a leukæmoid picture may suggest leukæmia, but spherocytosis and polychromasia affecting the red cells, if appreciated, will lead to the correct diagnosis. An eyepiece fitted with a micrometer scale and calibrated for use with the oil immersion lens should be kept on the observer's bench, it is of the greatest use. An impression of microcytosis or megalocytosis may be quickly confirmed or refuted by measuring the diameters of selected cells

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(2) [illegible]

(3)

(4)

(5)

supposed, but the results obtained thereby are of less diagnostic importance.* Nevertheless, it should not be forgotten that the focusing of attention on the variation in cell diameters in health and disease has, as has Wintrobe's advocacy of the measurement of mean cell volume, contributed considerably to the understanding and classification of the anæmias.

For clinical purposes the measurement of as many as 500 cells is superfluous, although this enables smooth diameter distribution curves to be obtained as a rule. The accuracy of the estimation of mean cell diameter is hardly affected by increasing the number of cells counted from 100 to 500; the standard deviation will, however, be reduced by counting the larger number of cells.

There exist at least two other possible methods for the measurement of red cell diameters in addition to Price-Jones' original technique and later variants of it; these are measurements by means of a micrometer scale introduced into the microscope eyepiece and the use of the diffraction (halometric) method, based on an entirely different principle. Brief descriptions of all three techniques are given below.

The Price-Jones Technique

The basic principle of this technique was the individual outlining with a sharp pencil of the images of red cells projected on to a white screen at a magnification of 1000 times. Usually 500 cells were outlined, and the outlines were measured later by means of a millimetre rule.

The following are brief details of the technique A well spread film is essential, the cells measured must lie free from each other and be free from distortion In order that the cells be clearly seen,

* [illegible]

CHAPTER 4

MEASUREMENT OF RED CELL DIAMETERS

NORMALLY, even in health, there is a readily appreciable variation in diameter between a population of red cells. It is largely owing to the work of Price-Jones (1) that this normal variation has been placed upon a quantitative basis. He showed that if the diameters of a large number of red cells were measured and the cells grouped together in classes according to their diameter, the frequency distribution curve of diameters was of the "normal" type. Price-Jones applied statistical methods to his data and worked out the limits of normal variation in great detail; he also demonstrated that characteristic deviations from the normal were encountered in various types of anæmia. This work excited great interest and at one time the drawing of a Price-Jones curve was deemed an almost essential step in the investigation of any obscure case of anæmia—although the labour expended contributed nothing to the understanding of the case and merely placed on paper what was to be seen by inspection of a stained film.

The measurement of red cell diameters in dried films is *undoubtedly a highly artificial method*; not only do cells shrink on drying, their diameters being 8–16 per cent less when dry, compared with their diameters in plasma (2a), but their apparent diameters will vary appreciably in different parts of a film, *and very careful selection of an area of film where the* cells are neither distorted in shape nor shrunken is required *before measurements should be undertaken*. Moreover, in an anæmia such as severe pernicious anæmia it is extremely *difficult if not impossible to measure accurately the many* abnormally shaped poikilocytes which are likely to be present.

In fact, not only does there seem little doubt that the laborious Price-Jones method of detailed measurement of red cells *in dried films* is less accurate than was at one time

supposed, but the results obtained thereby are of less diagnostic importance.* Nevertheless, it should not be forgotten that the focusing of attention on the variation in cell diameters in health and disease has, as has Wintrobe's advocacy of the measurement of mean cell volume, contributed considerably to the understanding and classification of the anæmias.

For clinical purposes the measurement of as many as 500 cells is superfluous, although this enables smooth diameter distribution curves to be obtained as a rule. The accuracy of the estimation of mean cell diameter is hardly affected by increasing the number of cells counted from 100 to 500; the standard deviation will, however, be reduced by counting the larger number of cells.

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the film should be deeply stained with eosin after fixation in methyl alcohol. It is convenient to place the microscope horizontally and by means of a prism attached to the eyepiece or by means of a plane mirror (5), so arrange it that the image is projected downwards on to a piece of white paper. A strong source of light is essential, and a darkened room is advantageous. The next step is to adjust the magnification to ×1000. In order to effect this, place on the moveable stage a micrometer scale divided into 1/100 mm and using the lower power objectives adjust the focus of the microscope so that the micrometer scale is projected and focused sharply on to the piece of paper and is in the centre of the field. Change to the oil immersion objective, and then superimpose on to the image of the micrometer scale a paper scale divided into millimetres. Readjust the distance of the paper from the prism or plane mirror until the projected image of 1/100 mm exactly corresponds to 10 mm of the paper scale. This gives a magnification of ×1000; 10 mm. of the image is then equal to 10μ. Replace the stage micrometer by the stained blood film and bring the image of the cells into sharp focus on the paper. Select a portion of the film where the projected images of the red cells are well separated, and not distorted. Use the 2/3" and/or 1/6" objectives to find such an area before employing the 1/12" lens. Outline 500 cells with a sharp pencil—without selection, but omitting cells at the periphery of the fields which are not in sharp focus.

When the drawing has been completed in perhaps one hour, remove the paper on which the outlines have been made from the table, and using a glass millimetre scale which can be closely applied to the outlined cells measure the greatest and smallest diameters of each cell to 0.5 mm., the mean of these two measurements is accepted as the diameter measurement in terms of μ. Write the calculated diameter within the drawing of each cell, as soon as it has been measured

e.g.	max. = 7.5 mm	max. 7.0 mm	max. 7.0 mm
	min. = 7.0 mm	min. – 6.0 mm	min. = 6.5 mm
	mean = 7.25 μ	mean = 6.5 μ	mean – 6.75 μ

When 500 cells have been measured group the diameters in intervals of 0.25 μ. The totals (frequencies) of each class interval may be plotted on squared paper as a distribution curve—"a grouped frequency distribution".

Various modifications of Price-Jones' technique have been proposed in order to save time and to avoid the errors introduced by first outlining the cells and then measuring the

outlines. It is possible to measure directly the images of the projected cells with a glass or celluloid rule and to write the mean of two measurements at right angles directly on to the cells' images on the paper. In this way the diameters of 500 cells may be measured and scored within an hour. At 2000 diameters a measurement of 15 mm. corresponds to 7.5 μ. Alternatively, a cell's diameter may be measured by seeing which fits the closest of a series of black rings of different sizes drawn on to the paper on which the images of the cells are projected (5). Another modification is that of Hynes and Martin (6) who, using the microscope in a vertical position, project the film on to a ground glass screen at a magnification of $\times 2000$. The diameters of the cells' images are measured by means of a celluloid protractor on which circles of a range of diameters are drawn.

Statistical Method for dealing with Measurements of Red Cell Diameters [after Price-Jones (1)]

The accompanying table is based on Price-Jones' procedure and shows how it is possible to calculate the arithmetic mean (M), the standard deviation (σ) and the coefficient of variation (V) of the diameters of the red cells of a sample of blood

Arithmetic Mean The class intervals are tabulated in column 1 and the corresponding frequencies of diameters in column 2 In order to obtain from the variable diameters the arithmetic mean for the sample of blood, the figures for the diameters in column 1 [illegible]

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one near the middle of the range (in the particular case given in the table, 6 75 μ); then mark off in column 3 the deviations or distances in class intervals from this arbitrary mean; multiply columns 2 and 3, i e the frequencies corresponding to the deviations, and ad [illegible]

468 values le [illegible]

is +418−46 [illegible]

the differen [illegible]

−50/500=−0.1 in $\frac{1}{4}$ μ units The true mean is thus 6 75 μ−0.1 ($\frac{1}{4}$ μ)=6.75 μ−0 025 μ=6 72 μ

Standard Deviation The standard deviation (σ) is the measure in μ of the scatter or dispersion of the diameters, their range in

Table VI

Statistical Method for Dealing with Measurements of Red Cell Diameters

[After Price-Jones (1)]

1 Mid point of class intervals (in $\frac{1}{4}\mu$ units)	2 Frequencies	3 *Deviation from arbitrary mean (6.75 μ) in class interval units*	4 Product (2 × 3)	5 Product (2 × 3²)
5.5	10	−5	50	250
5.75	18	−4	72	288
6.0	42	−3	126	378
6.25	70	−2	140	280
6.5	80	−1	80	80
6.75 arbitrary mean = A)	83	0	−468	
7.0	82	+1	82	82
7.25	51	+2	102	204
7.5	33	+3	99	297
7.75	20	+4	80	320
8.0	11	+5	55	275
			+418 −468 = −50	$\frac{2354}{500}$ = 4.71

$$M(\text{true mean}) - A\ (\text{arbitrary mean}) = \frac{-50}{500}\ (\tfrac{1}{4}\mu)$$

$$= -0.1\ (\tfrac{1}{4}\mu)$$

$$= -0.025\mu$$

$$M = 6.75 - 0.025\mu$$

$$= 6.72\mu$$

$$\sigma^2 = 4.71 - (M - A)^2\ \tfrac{1}{4}\mu$$

$$= 4.71 - 0.01\ \tfrac{1}{4}\mu$$

$$= 4.7\ (\tfrac{1}{4}\mu)$$

$$\sigma = 2.17\ (\tfrac{1}{4}\mu)$$

$$= 0.54\mu$$

$$V = \frac{\sigma}{M} \times 100$$

$$= \frac{0.54 \times 100}{6.72}$$

$$= 8.0\%$$

The patient whose red ...

size, and the way in which the numerical frequencies of the different diameters are arranged. The scatter about the mean implies diameters less (−) and diameters greater (+) than the mean diameter. In order to obtain some quantity that shall vary with the general dispersion, it is necessary to average the deviations by a process that treats them as if they were all of the same sign, and squaring is the simplest process for this purpose.

Column 5 gives values for the squared deviations (column 3 multiplied by column 4). Addition of these products gives 2354, which divided by 500=4.71. The difference between the mean (M) and the arbitrary mean (A) is −0.1. The square of this value is 0.01. This subtracted from 4.71 gives 4.70 as σ^2; σ (the standard deviation) is thus 2 17 in $\frac{1}{4}$ μ units or 0.54 μ.

Coefficient of Variation. The coefficient of variation (V) measures the degree of scatter of the distribution in relation to the mean value of the thing varying It is the standard deviation (σ) expressed as a percentage of the mean ($V = \frac{\sigma \times 100}{M}$) and forms a measure of variability which is independent of the unit in which the measurements have been made.

In the example given $V = \frac{0.54 \times 100}{6.72} = 8.0$ per cent.

The diameter distribution curve based on the above data is illustrated in Fig. 5

Normal Ranges (Dry films)	Price-Jones (1).
Mean cell diameter (M.C.D.)	6.7 μ to 7.7 μ (Mean 7.2 μ)
Coefficient of variation (V)	5.3 to 7.3% (Mean 6.3%)
Standard deviation (σ)	0.35 μ to 0.56 μ

Measurement of Red Cell Diameters by the Diffraction Method

The diffraction method for measuring the size of small objects was first applied to the measurement of the diameters of red blood cells by Pijper in 1919. The principle of the method is that when a parallel beam of polychromatic light is passed through a film of relatively opaque red cells the light is diffracted by each individual red cell, the degree of diffraction varying with the cell diameter. The individual spectra appear to the human eye as a concentric compound spectrum surrounding the source of light; they may be resolved as an image on a screen by means of a convex lens.

Each diffraction pattern consists of a bright centre, surrounded by an orange ring, and then the spectral colours in their natural sequence from violet to red. Outside the red ring fainter green and red circles may be seen.)

The size of the concentric spectral rings varies inversely with the diameters of the red cells and directly with the distance of the film from the light source. In macrocytic anæmias the spectral circles are smaller than normal and in

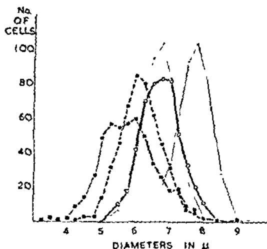


FIG. 5 RED CELL DIAMETER DISTRIBUTION CURVES (PRICE JONES CURVES)

O——O is a curve constructed from the blood of a mild case of familial hæmolytic anæmia (acholuric jaundice)—see note added to legend to Table VI ●— — — —● and ■══════■ are curves drawn from blood from two typical cases of familial hæmolytic anæmia The characteristic microcytosis is well demonstrated The thin lines indicate the maximum and minimum normal curves [data from Price-Jones (1)]

microcytic anæmias they are larger. The distance between the violet and red circles gives an expression of the degree of anisocytosis

As the spectral rings are produced by the blending of rings of different sizes depending upon the varying diameters of the red cells, the smaller the degree of anisocytosis the narrower and clearer are the bands, conversely in the case of a blood showing much variation in cell size the spectral pattern is

much less clear. Nevertheless, measurement of cell diameter by the diffraction method has been widely used in clinical hæmatology, and though the method is hardly precise, with suitable apparatus it is not more inaccurate than the measurement of the images of projected red cells. It has the great advantage of being extremely quick. A well spread film is required, and in the area examined the red cells should be undistorted and just not overlapping If the film is too thinly made, the spectra are weak in intensity. It is best to examine an unstained film.

The theory and the use of the diffraction method of measuring the diameters of the red blood cells is considered at length by Ponder (2b), and the difficulty of obtaining exact measurements stressed The use of monochromatic light is recommended. By converting the disc-like red cells into spheres by means of a sphering agent such as distearyl lecithin, Ponder has estimated mean cell volume by means of the diffraction technique. The results agree closely with those obtained by the hæmatocrit method.

Several types of apparatus have been designed In that of Pijper (7) the spectral image produced by the film to be tested is projected on to a screen alongside that of a normal control film so that the size of the coloured rings can be compared. In addition, it is possible to calibrate the screen by means of a formula (7) or

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their apparatus

monochromatic light is obtained by introducing a green filter with the result that the orange spectral band appears as a black ring This is viewed through an eyepiece into which is inserted a micrometer scale calibrated to 0 1 mm In this way the diameter of the black ring can be measured and the corresponding mean cell diameter calculated from a formula

Another method suggested is that of Emmons (9) In his apparatus, the eriometer, a central light source surrounded by a ring of fine holes illuminated from behind is viewed through a blood film and the distance between the film and the light source adjusted until the red spectral ring is superimposed upon the concentric ring of illuminated holes The apparatus is calibrated so that the mean red cell diameter can be calculated from the distance between the film and source of light under the above conditions

Other techniques which may be mentioned, are those of Pryce

Interesting comparisons between the results obtained by diffraction methods and by direct measurement by the Price-Jones projection technique or by the use of an eyepiece micrometer are given in the papers of Haden (12) and Pijper (7). Haden found that Emmons' eriometer and Pijper's blood cell tester gave the best results.

Unquestionably the diffraction method is a useful way of estimating red cell diameters, and the experienced observer will be able to appreciate not only alteration in mean cell diameter but also, depending upon the clarity and width of the spectral bands, the degree of anisocytosis which may be present.

It is more doubtful whether the information obtained by use of diffraction methods is often of decisive use in clinical hæmatology. Possibly in the follow up of patients with macrocytic anæmias the method may call attention to persistent macrocytosis. This is, however, as likely to be obvious to the experienced eye, aided perhaps by an eyepiece micrometer, when the film is viewed down the microscope. Certainly, the diffraction method of measuring diameters is no substitute for microscopy, which reveals so much besides, it is a useful accessory method, but in clinical practice it can be dispensed with

Measurement of Red Cell Diameters with the aid of an Eyepiece Micrometer

The eyepiece micrometer scale can be usefully employed in the measurement of cell diameters It has the advantage of directness and simplicity and can be quickly applied. Where a relatively large number of cells are to be measured, it is, however, simpler and quicker to project them at a magnification of $\times 1000$ or $\times 2000$ and to measure the images by one of the methods previously described

The scale of the eyepiece micrometer has to be calibrated in relation to the objective, eyepiece and tube length employed,

before it can be used. This may be done using a slide on which a scale, usually 1 mm. in 0.01 mm. (10 μ) intervals, has been engraved, or alternatively, the calibrations on a counting chamber (the side of a smallest square is 0.05 mm. in length).

It is convenient to have a conversion scale kept near the microscope, e.g. using a 1/12 in. objective and ×6 eyepiece,

5.0	divisions	= 6.6 μ
5.5	,,	= 7.2 μ
6.0	,,	= 7.9 μ
6 5	,,	= 8.5 μ, etc.

The diameters of red cells can be measured to about 0.5 μ without difficulty with the aid of the eyepiece micrometer. Although minor degrees of deviation from the normal will not be detected, the method is very useful, and in practice it is possible by measuring a few cells whose diameters are representative to confirm or refute visual impressions of abnormalities in cell size This does not mean that the observer should search for the largest or smallest cells to measure; a few as large as 9 μ or as small as 6 μ may be found in normal blood It is much more significant to find an unusually high proportion of cells of 8 5 μ than a few cells outside these limits Price-Jones' (1) ranges in health (±3 σ) give the outside normal limits for cells of the dimensions just referred to

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(5) Humble, J G, and Belyavin, C (1948) "A simplified Price-Jones technique" *J clin Path*, 1, 77

(6) Hynes, M, and Martin, L C (1936) "A rapid method of measuring erythrocyte diameters" *J Path Bact*, 43, 99

(7) Pijper, A (1947) "The diffraction method of measuring red blood cells" *J Lab clin Med*, 32, 857

(8) Tzanck, A, and Bessis, M (1947) "Un nouvel hémo-diffractomètre" *Sang*, 18, 71

Other techniques which may be mentioned, are those of Pryce (10) and Bock (11) in which two sources of light are viewed through a film and the distance between the film and light sources adjusted until the red spectral rings of each halo just touch. Long even films are required, and these are difficult to spread.

Interesting comparisons between the results obtained by diffraction methods and by direct measurement by the Price-Jones projection technique or by the use of an eyepiece micrometer are given in the papers of Haden (12) and Pijper (7). Haden found that Emmons' eriometer and Pijper's blood cell tester gave the best results.

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The scale of the eyepiece micrometer has to be calibrated in relation to the objective, eyepiece and tube length employed,

Chapter 5

SUPPLEMENTARY STAINING AND OPTICAL TECHNIQUES

Examination of living blood cells. Supravital staining and examination by phase contrast. Tests for sickle cells. The peroxidase reaction. Additional

EXAMINATION OF BLOOD CELLS SUSPENDED IN PLASMA

The examination of a drop of blood sealed between a slide and coverglass is a useful procedure and may reveal information of diagnostic importance.

The preparation may be examined in several ways, by ordinary illumination, with or without the addition of dyes such as Janus green and neutral red (supravital staining), by dark ground illumination or with the aid of a phase contrast condensor Chemically clean slides and cover-slips should be used and a thin film made between them If the glass surfaces are free from dust, the blood should spread out spontaneously and no pressure should be needed, indeed this is undesirable The edges of the preparation should be sealed with a melted mixture of equal parts of vaseline and paraffin wax.

In an unstained preparation examined with ordinary or dark ground illumination rouleaux formation is usually seen in varying degree, the abnormal irregular rouleaux seen in hæmolytic anæmias associated with marked spherocytosis is characteristic Blood parasites such as microfilariæ and spirochætes such as Borrelia recurrentis may be seen without difficulty, the presence of small numbers of the latter being revealed by occasional slight agitation of groups of red cells Similarly, sickling of red cells may be revealed (see later) If

(9) EMMONS, W. F. (1927-28). "The clinical eriometer." *Quart. J. Med.*, 21, 83.

(10) [illegible]

(11) [illegible]

(12) [illegible]

minimum. Too high a concentration impairs motility and ultimately kills the cells and stains their nuclei. Where many immature leucocytes are present, as in leukæmia, or in bone [illegible]tions may be necessary [illegible] of the film of blood will [illegible] the dyes.

[illegible] phil granules are stained yellowish, those of eosinophils orange red and those of basophils maroon. Vacuoles may take up the stain; they appear as bright red spheres of varying size. They may increase in size and number during observation

Motility. The neutrophil polymorphs should be actively motile if the preparation is kept at 35° to 39°C. on a warm stage and the dye concentrations are correct Streaming of their somewhat refractile granules can be readily seen Normal monocytes may show just perceptible movement. Other cells usually show no movement in this type of preparation, though motility of lymphocytes, etc., can be observed in hanging drop preparations (4) Light is inimical to motility; the microscope lamp should thus be switched off when not required. In the leukæmias the primitive cells as a rule exhibit little movement; in monocytic leukæmia, however, actively motile cells may be seen.

*Mitochondria** are seen in their largest numbers in immature cells; in the *myeloblast* they are very numerous and fine and may be scattered diffusely or in several groups, in *myelocytes* they diminish in number as the cells mature, often occupying one or more sectors of the cell A few may be seen in "young" metamyelocytes; they are absent in adult polymorphs. The mitochondria of the *lymphoblasts* are relatively few in number, they are distinctly larger than those seen in myeloblasts and myelocytes and may be recognizably rod-like They diminish in numbers as the cells mature, but are definitely present and easily seen in mature lymphocytes.

In the monoblast the mitochondria are said to be fine and numerous, in the promonocyte and adult monocyte they are fairly numerous, but variable in size.

Specific Granules * The staining reactions of the granules of the polymorphs (yellow), eosinophils (orange red) and basophils (maroon) have already been mentioned. Rose-coloured fine granules appear in the early types of promyelocytes and increase in number as the cells mature They are most numerous in the "type B" of Sabin and may be closely packed in a large sector, in the more adult cells they are gradually replaced by the yellowish staining granules of the adult polymorphs

* Good illustrations of blood cells stained supravitally by Janus green and neutral red are given in Whitby and Britton's book (5)

a warm stage is available, the motility of the leucocytes can be studied; usually only the granulocytes show significant progressive movement. Whitby and Hynes (1) describe the appearances of leucocytes under dark ground illumination; cytoplasmic particles such as granules and mitochondria can be readily seen. Most workers have, however, studied preparations stained supravitally (2, 3), and a description of this technique is given below.

Supravital Technique

Chemically clean glassware is essential. The following stains are required.

(A) A saturated solution of neutral red in absolute ethyl alcohol.

(B) A saturated solution of Janus green in absolute ethyl alcohol

Before use add 0.2 ml. of (A) to 5 ml. of absolute ethyl alcohol, and deliver 2×1.5 ml. volumes of the diluted stain into separate test tubes. It is convenient to work with at least two concentrations of Janus green. Therefore, add 0.02 ml. and 0.05 ml of solution (B) to each of these 1.5 ml. volumes.

Preparation of Slides. Deliver by pipette several drops of the stain mixture on to a clean slide so as to flood its surface with the dyes, drain off the excess back into the tube and allow the slide to dry rapidly by waving it over a heated wire gauze In this way an even deposition of the dye may be obtained. When dry, slides may be stored until required: they must be protected from dust

Setting up the Specimen. A small drop of freshly drawn blood, taken directly from a skin puncture, from a vein or from the marrow cavity, is placed on to one or more prepared slides of each dye concentration and covered by a coverslip so as to obtain a moderately thin film The edges of the preparation are sealed with vaseline-paraffin wax mixture.* Staining is slow and may need twenty to thirty minutes, it is hastened by keeping at 37°C.

Janus green which stains the mitochondria blue is the more toxic of the dyes, its concentration, therefore, has to be kept to a

* Equal volumes of vaseline and paraffin wax melted together.

minimum Too high a concentration impairs motility and ultimately kills the cells and stains their nuclei Where many immature leucocytes are present, as in leukæmia, or in bone [illegible] y be necessary [illegible] of blood will

Neutral red is much less toxic; neutrophil granules are stained yellowish, those of eosinophils orange red and those of basophils maroon. Vacuoles may take up the stain, they appear as bright red spheres of varying size They may increase in size and number during observation.

Motility. The neutrophil polymorphs should be actively motile if the preparation is kept at 35° to 39° C on a warm stage and the dye concentrations are correct Streaming of their somewhat refractile granules can be readily seen Normal monocytes may show just perceptible movement Other cells usually show no movement in this type of preparation, though motility of lymphocytes, etc., can be observed in hanging drop preparations (4). Light is inimical to motility, the microscope lamp should thus be switched off when not required. In the leukæmias the primitive cells as a rule exhibit little movement, in monocytic leukæmia, however, actively motile cells may be seen.

*Mitochondria** are seen in their largest numbers in immature cells; in the *myeloblast* they are very numerous and fine and may be scattered diffusely or in several groups, in *myelocytes* they diminish in number as the cells mature, often occupying one or more sectors of the cell A few may be seen in "young" metamyelocytes; they are absent in adult polymorphs The mitochondria of the *lymphoblasts* are relatively few in number; they are distinctly larger than those seen in myeloblasts and myelocytes and may be recognizably rod-like They diminish in numbers as the cells mature, but are definitely present and easily seen in mature lymphocytes

In the monoblast the mitochondria are said to be fine and numerous; in the promonocyte and adult monocyte they are fairly numerous, but variable in size

Specific Granules * The staining reactions of the granules of the polymorphs (yellow), eosinophils (orange red) and basophils (maroon) have already been mentioned. Rose-coloured fine granules appear in the early types of promyelocytes and increase in number as the cells mature. They are most numerous in the "type B" of Sabin and may be closely packed in a large sector; in the more adult cells they are gradually replaced by the yellowish staining granules of the adult polymorphs.

* Good illustrations of blood cells stained supravitally by Janus green and neutral red are given in Whitby and Britton's book (5)

Vacuoles staining bright red may be seen as small spheres in most adult lymphocytes; in monocytes they are characteristically present in rather large numbers. They vary in size and are present throughout the cytoplasm; a definite group (rosette) may be seen in the concavity (hof) of the nucleus. Similar vacuoles are seen in promonocytes and monoblasts.

Erythroblasts. Both fine mitochondria and red vacuoles may be seen in developing red cells. They diminish in number as the cells mature and the nuclei undergo pyknosis. Mitochondria are not seen in the adult red cell; an occasional red vacuole may, however, be encountered.

The use of the phase contrast condenser has opened up new possibilities for the examination of unfixed and living tissues, and the equipment will no doubt be widely used in hæmatology when it becomes more freely available. At the moment it is too early to assess its practical implications, but already its use has clearly demonstrated that mitochondria are responsible for the pale unstained areas so commonly seen in the cytoplasm of primitive cells, particularly close to the nuclear membrane (6), and that the apparent nuclear pattern as seen in stained films is to some extent modified by the cytoplasmic structures that overlie it (7).

It is clear also that the use of phase contrast condenser will enable the observer to view living cells and to observe their cytoplasmic structures without the necessity and disadvantage of using potentially toxic dyes to outline them. In addition the nuclear chromatin and nucleoli of unstained cells can be visualized.

VALUE OF SUPRAVITAL STAINING AND EXAMINATION BY THE PHASE CONTRAST TECHNIQUE

These are primarily research techniques and perhaps are seldom of much practical importance. In the case of supravital staining considerable care is needed to obtain good results, and both techniques require experience in interpretation. However, in addition to enabling observations of scientific rather than practical importance to be made, use of these techniques may help to differentiate the various

types of acute leukæmia by providing more information than can be obtained by Romanowsky or peroxidase staining. It is possible that, although in the past this has always been considered a purely academic problem, the use of folic acid antagonists in the treatment of leukæmia (8) will increase the importance of accurate cytological diagnosis.

TESTS FOR SICKLING OF THE RED CELLS

A brief account of tests for sickling of red cells is given below, although the phenomenon is rarely seen in this country. The recognition of this abnormality is of great importance in a country such as the United States in which there is a large negro population.

The onset of sickling of susceptible cells is determined by the presence within the cells of hæmoglobin in a reduced state. The proportion of reduced hæmoglobin necessary to induce sickling varies from case to case; if the phenomenon occurs at physiological levels of reduced hæmoglobin, "sickle cell anæmia" may result. A "sickling trait" of no clinical importance is much more frequently encountered; in these cases sickling takes place *in vitro*, but not *in vivo*, at concentrations of reduced hæmoglobin

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Daland and Castle (16) recommend adding a drop of 2 per cent aqueous sodium bisulphite to a drop of blood on a slide, mixing and sealing a thin film under a coverslip. Under these circumstances sickling may be seen within 15 minutes at room temperature.

ADDITIONAL STAINING METHODS

The Peroxidase Reaction applied to Blood Films

This reaction depends upon the presence of enzymes (peroxidases) within the cytoplasm of leucocytes of the granulocyte series and to a lesser extent of monocytes. Blood or marrow films are treated with benzidine and hydrogen peroxide after fixation, and the benzidine is oxidized by

the peroxide in the presence of peroxidase. Sodium nitroprusside which forms a blue compound with oxidized benzidine may be added to the benzidine, but is not essential.

Solutions Required: 1. *Fixative.* Absolute ethyl alcohol nine parts; neutral formalin (40 per cent formaldehyde) one part

2. A saturated aqueous solution of *benzidine.*

3. A saturated aqueous solution of sodium nitroprusside. Before use one part of (3) is added to 99 parts of (2).

4. A freshly made solution of hydrogen peroxide "10 vols." Two drops (0.04 ml.) are added to 10 ml. of distilled water.

Method. Fresh air-dried films are required. These are fixed for one minute in the formol-alcohol fixative solution, washed in distilled water and set upright to dry. Add one part of the peroxide solution to two parts of the nitroprusside-benzidine solution, mix and flood the slide with the mixture for one minute. Wash in water and examine under the microscope If the staining is satisfactory, the granules in the polymorphs should be stained a deep blue black. The preparation may be counterstained with 0.1 per cent aqueous safranin for one minute or in dilute (1:20) Giemsa for seven to ten minutes.

The Value of the Peroxidase Reaction

Developing and adult granulocytes give a positive staining reaction, but in the earliest myeloblasts the result is usually negative Positive granules are usually present in small numbers in promonocytes and in monocytes. The results with lymphocytes are uniformly negative in both adult and immature cells.

The peroxidase reaction is an interesting phenomenon, but not often of much practical value Occasionally, a positive reaction may help to distinguish between myeloblasts and lymphoblasts, but as the most immature myeloblasts give a *negative reaction, the test breaks down just when it is most* needed

Unna-Pappenheim Stain

The Unna-Pappenheim combination of pyronin with

methyl green can be used to stain both dry or wet-fixed films of peripheral blood or aspirated bone marrow. Wet-fixed films give the more satisfactory results. The pyronin stains cell components containing ribonucleic acid bright red, e g. the nucleoli and the cytoplasm of all types of primitive cells and of mature cells, such as plasma cells, in which the ribonucleic acid persists The methyl green stains the chromatin greenish black.

The following technique gives good results (details kindly supplied by Dr. J. C White) The films are placed as soon as they are made in Susa's* fluid and "wet-fixed" for one hour. After fixation they are treated with Gram's iodine solution (5 min) followed by 1 per cent sodium thiosulphate solution (5 min.), washed well in running water and finally rinsed in distilled water. The films are then stained for one hour in a solution of the following composition —

Pyronin (B D H)	0 3 g
Methyl green	0 7 g
Glycerin	20 ml
Absolute ethyl alcohol	2 5 ml
0.5 per cent phenol in distilled water to 100 ml.	

The stains are ground in the glycerin and alcohol in a mortar and the phenol water added later The solution is boiled for 2 minutes, then filtered

After staining, the slides are rinsed in distilled water and dehydrated in tertiary-butyl alcohol, cleared in xylol and mounted in neutral balsam or Gurr's medium If differentiation is required, a rapid dipping into absolute ethyl alcohol, in which pyronin is very soluble, suffices Dehydration in the butyl alcohol is slow, but little stain is abstracted.

This stain is particularly useful in demonstrating nucleoli In wet-fixed well stained films they appear as one or more rounded or elongated red-staining structures † Illustrations are given in White's paper (9)

* T[illegible]

[illegible] 4 ml
[illegible] 20 ml
[illegible] 80 ml

† In dry films the nucleoli are less well demonstrated by the above technique In certain respects wet-fixation has definite advantages over dry-fixation Delicate cells and structures are often better preserved, and in conjunction with ribonuclease the stain can be used as a histochemical test In wet-fixed films leucocytes are more globular, i e more nearly the shape in life The red cells are as a rule badly distorted

Feulgen's Stain

The Feulgen reaction is generally held to be a specific cytochemical test for desoxyribonucleic acid, an important component of nuclear chromatin. When applied to films of marrow or peripheral blood, the nuclei of the adult cell types stain most intensely, and the nuclei of the primitive cells least intensely. The nucleoli do not stain. Howell-Jolly bodies stain by this method, i.e. are Feulgen-positive, and chromosomes stain particularly well. The stain is thus well suited to the study of mitoses.

The best staining results are obtained after wet-fixation with Susa's fluid, but satisfactory staining of dry-fixed films can be achieved. In a well stained preparation the nuclear chromatin is sharply stained shades of purple. The results of Feulgen staining are illustrated by Gardikas and Israëls (10).

The following technique based on that of Bauer* gives good results Fix films of peripheral blood or marrow in Susa's fluid for one hour Wash and treat with iodine and sodium thio-

in distilled water The exact time of hydrolysis needs to be determined by experiment; with films fixed in Susa, 4 min. seems to be the optimum. Stain for two hours in Feulgen's reagent This is prepared by dissolving 1 g of basic fuchsin in 200 ml. of boiling distilled water, then filtering when the solution has cooled to about 50°C, and when cooled to about 25°C., adding 20 ml. N.HCl and 1 g anhydrous sodium bisulphite Sulphur dioxide is evolved and the solution turns yellow, it should be kept in a well stoppered bottle in the dark and not used for at least 24 hours after preparation The slides are then washed for 15 min. in three successive jars of the following solution; 10 ml 10 per cent aqueous solution of anhydrous sodium bisulphite, 10 ml N HCl, distilled water to 200 ml Finally, the slides are well washed in running water for 10 min. rinsed in distilled water and dipped for a few seconds in a 1 per cent aqueous solution of light green, which is used as a counterstain.

The chemistry of the Feulgen reaction is complex and has been the subject of some controversy Recent views are given in a paper by Overend and Stacey (11)

* See "The Microtomist Vade-Mecum" (1937), 10th Ed, p 259-61. Churchill London

Staining with Sudan Black

Sudan black B. has been used by Sheehan (12) and later by Bailif and Kimbrough (13) and others to stain the granules of leucocytes, many of which appear to contain lipoid. The results indicate a close parallelism between sudanophilia and a positive peroxidase reaction, and sudan staining may perhaps be used as a substitute for the enzyme reaction Further details and references to the literature are given in the above mentioned papers, and by Rheingold and Wislocki (14).

HEINZ BODIES IN RED CELLS

Inclusions within adult red cells developing as the result of the action of phenylhydrazine were first fully described by Heinz in 1890 and are now generally referred to as "Heinz bodies."

Since Heinz's description it has been found that many types of chemicals, including the sulphonamides and aniline derivatives cause the appearance of Heinz bodies. Their production is often but not invariably associated with the production of methæmoglobin. The chemistry of Heinz bodies, their derivation and the site of their formation is still not fully understood . it appears that they consist of denatured globin and that they are derived from hæmoglobin. They are not usually seen in reticulocytes.

The whole problem of Heinz body formation has been recently well reviewed by Webster (15)

Method of Demonstration

Unstained Preparations. Heinz bodies may be seen as refractile objects in dry unstained films, if the illumination is cut down by lowering the microscope condenser. They are also easily seen by dark ground or phase contrast illumination. The size of the particles varies from 1–2 μ to half the size of the corpuscle. One or more may be present in a single cell. They are usually close to the cell membrane, but may move around within the cell in a slow Brownian movement.

Stained Preparations. Methyl violet, recommended by Heinz himself, stains the bodies excellently.

Equal volumes of blood and 0.5 per cent methyl violet in normal saline are mixed together, and after standing for about

ten minutes at room temperature, films may be made or the suspension of corpuscles viewed between slide and cover glass. The Heinz bodies stain an intense purple. They also stain with other basic dyes. In the case of brilliant cresyl blue they stain less intensely than with methyl violet. They may, however, be readily seen in a well stained reticulocyte preparation.

If permanent preparations are required, the vitally stained films should be fixed by exposure to formalin vapour for 5–10 minutes. If fixed in methyl alcohol, the bodies are decolorized. Formalin-fixed films may be counterstained with 0.1 per cent eosin or 0.1 per cent safranin after washing well in distilled water. Because of the fixing effect of methyl alcohol, Heinz bodies are not seen in films stained by Romanowsky dyes and their presence may be missed for this reason

Significance of Heinz Bodies in Clinical Hæmatology

The presence of Heinz bodies is a sign of chemical poisoning and they should be looked for in any case of hæmolytic anæmia where it is possible that a chemical toxic agent is implicated or where the origin of the disorder is obscure*. In chemical workers a search for Heinz bodies is a simple ancillary method for controlling exposure to potentially dangerous chemicals.

THE STAINING OF SIDEROCYTES

Siderocytes, or red blood cells containing granules of iron which give a positive Prussian blue reaction were described by *Gruneberg* (18, 19) in small numbers in the blood of normal rat, mouse and human embryos, and in large numbers in mice with a congenital anæmia. They have since been detected in adult human blood, chiefly after splenectomy, sometimes in large numbers. They may be found in nucleated as well as in non-nucleated red cells, but only in the later normoblasts in the cytoplasm of which hæmoglobin is being formed Their presence seems to depend on some abnormality of hæmoglobin formation. The granules also stain by Romanowsky dyes. Details are given by McFadzean and

Davis (20) and Dacie and Doniach (21). They have been detected in experimentally produced lead poisoning (22).

Method of Staining

Air dried films are fixed with methyl alcohol for two to five minutes. When dry, the slides are flooded with a solution of 1 per cent potassium ferrocyanide in N/10 HCl made by mixing equal volumes of 2 per cent potassium ferrocyanide* and N/5 HCl immediately before use.

After two minutes the slides are well washed in distilled water and may then be weakly counterstained with 0.1 per cent aqueous safranin for a few seconds.

Siderocytes contain one or more (rarely many) iron-containing unevenly distributed granules, in size varying from about 1 μ down to the limits of visibility.

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* It is desirable to make up a fresh solution of potassium ferrocyanide each time the stain is used

ten minutes at room temperature, films may be made or the suspension of corpuscles viewed between slide and cover glass. The Heinz bodies stain an intense purple. They also stain with other basic dyes. In the case of brilliant cresyl blue they stain less intensely than with methyl violet. They may, however, be readily seen in a well stained reticulocyte preparation.

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CHAPTER 6

BONE MARROW BIOPSY

Techniques and methods of examination. The normal "myelogram." The preparation and staining of histological sections.

BIOPSY of bone marrow is an indispensable adjunct to the study of the diseases of the blood and may be the only way in which a correct diagnosis can be made (1, 12). The method of trephining has been largely superseded, but not quite replaced, by Arinkin's technique of needle biopsy and variants of it. Needle biopsy has in fact many advantages over the trephine method; it is simple and safe, relatively painless, can be repeated many times and even performed on out-patients. Not only will it provide material for making films, but quite good sections of aspirated marrow fragments may be obtained. Trephine biopsy has the disadvantages of being a surgical operation which may be dangerous in the presence of a bleeding tendency or severe granulocytopenia; it can seldom be repeated. However, it is capable of giving decisive information when methods of aspiration fail, as they may in cases of myelofibrosis, marrow hypoplasia or occasionally in leukæmia. Marrow puncture seems to be safe in almost all circumstances, even in thrombocytopenic purpura. It should, however, never be attempted in hæmophilia or in a like disorder where the coagulation time is prolonged

THE TECHNIQUE OF NEEDLE BIOPSY OF THE MARROW

Sternal Puncture. The usual site for marrow puncture is the manubrium or the first or second pieces of the body of the sternum., but they are not the only possible sites (see later). The manubrium of the sternum in general seems to give a

(14) Reingold, J. J., and Wislocki, G. B. (1948). "Histochemical methods applied to hematology." *Blood*, 3, 641.

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(16) ... ple and rapid method ... the use of reducing

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(20)

(21)

(22) ... with reference to the effect

(23)

3, 387

spinous processes is particularly convenient, as they lie superficially and the overlying tissues are not highly sensitive. The needle is passed vertically into the spines of the lumbar or lower thoracic vertebræ of the sitting or reclining patient (3). Rather more pressure is required than for sternal puncture. An added advantage is that the patient cannot see what is happening and that several attempts at puncture and aspiration can be made in the same anæsthetized area if necessary.

Iliac Crest Puncture. The iliac crest is another site from which active marrow may be withdrawn (4). The needle is passed vertically (i.e. downwards and medially) into the cavity of the ilium from just posterior to the anterior superior iliac spine. As with spinous process puncture the bone is often appreciably harder to pierce than is the sternum, and a stout needle is required. Although there is normally considerable variation in the composition of cellular marrow withdrawn from adjacent or different sites, the general trend and type and maturity of hæmopoiesis and the balance between erythropoiesis and leucopoiesis is similar (5).

In practice it is a distinct advantage to have a choice of several sites for marrow puncture, particularly in the case of a "dry" puncture or when blood alone is withdrawn. In these cases aspiration at a different site may yield cellular marrow or strengthen suspicion of a widespread change such as fibrosis or aplasia.

Bone Marrow Biopsy in Children

In the youngest children from birth to four to five years the medial aspect of the head of the tibia may be punctured and active marrow withdrawn. This procedure is certainly less dangerous and less upsetting to the child than is sternal puncture. In older children the tibial cortical bone is usually too dense and the marrow within normally less active. Better samples are obtained by sternal puncture, but particular care and experience are required. The dimensions of the marrow cavities in the sternum and the thickness of the cortical bone in childhood are described by Diwany (6). It is likely that iliac crest puncture will prove to be a satisfactory substitute.

slightly better marrow sample than sites in the body of the bone, although in the former it may be less easy to be certain that the needle is in the cavity of the bone. *If serial punctures are being performed, a different site should be selected for* each puncture in order to avoid marrow possibly disorganized by hæmorrhage resulting from previous punctures.

A special needle is used. This should be short and stout with a well fitting stilette, and must be provided with an adjustable guard. The patient lies on his back in a semi-recumbent position and the skin covering the upper part of the sternum is cleansed with spirit or iodine. The skin, subcutaneous tissue and periosteum overlying the site of the puncture are carefully anæsthetized with novocain or equivalent. The guard on the needle is adjusted to about ½ in. from the needle point, depending on the thickness of the subcutaneous tissue. The needle is then pushed by a boring motion into the cavity of the bone. The amount of force required varies, but may need to be considerable. It is usually easy to appreciate when the cavity of the bone has been entered. The stilette is then removed and a well fitting *1 or 2 ml. syringe used to suck up not more than 0.2 ml.* of marrow contents—bone marrow diluted with a variable amount of blood. Usually material may be sucked into the syringe without difficulty; occasionally it may be necessary to reinsert the stilette and push the needle in a little further and suck again. If further attempts at aspiration prove unavailing, it is worth while trying the effect of greater suction, a *5 or 10 ml. syringe may be used for this purpose.* Films should be made from the aspirated material without delay (see p. 78). The remainder of the material may then be delivered into methyl alcohol-formalin fixative and sections prepared as described by White, Baker and Griffin (2). The films should be stained without delay by the May-Grunwald-Giemsa technique, or fixed for five minutes in absolute methyl alcohol *if it is desired to keep them unstained for longer than* a few hours.

Spinous Process Puncture. Recently, the spinous processes of the vertebræ have been punctured and found in adults to yield good samples of marrow. Puncture of the

Quantitative Cell Counts on Aspirated Marrow. A number of figures for the cell content of aspirated normal marrow have been given in the literature (8, 9) The variation is extremely wide. This is hardly surprising, in view of the uncontrollable factor of dilution with peripheral blood, and the tendency of the marrow cells to adhere together in clumps of varying size

For the above reasons quantitative cell counts seem hardly worth carrying out. The degree of cellularity can be assessed within broad limits as increased, normal or reduced by inspection of a stained film, and for practical purposes this seems all that is necessary.

Qualitative Cell Counts on Aspirated Marrow; the "Myelogram"

Most workers perform differential counts on marrow films and by presenting the data in the form of a myelogram express the incidence of the various cell types as percentages. Such figures are unfortunately not as accurate as they might appear to be It must not be forgotten that smears made from aspirated material include cells from peripheral blood as well as from the marrow, and that the varying dilution with blood involves an error for which no compensation is possible In addition, the more fixed and primitive cells, and large cells such as megakaryocytes, tend to resist aspiration and remain in the marrow Those which are aspirated tend to be most irregularly distributed, most are carried to the tail of the film.

Pontoni (10) introduced the term "hæmomyelogram" for a differential count performed on aspirated material and restricted the use of the "myelogram" to differential counts made when fully ripened leucocytes are excluded, a more correct usage His leuco-erythrogenetic ratio based on his "myelogram is similarly a better expression of the relative proportions of leucopoiesis to erythropoiesis than is the more commonly used leucocyte-erythroblast ratio which is based on counts from which mature leucocytes are not excluded Further details are given in Dacie and White's (1) review

Ideally, differential counts should be performed on sectioned material, but the much greater difficulties in identification make this impractical. Dameshek *et al* (11) give interesting figures for parallel observations made on sections and on smears, and other observations are given in Osgood and Seaman's excellent review (8). Osgood and

THE EXAMINATION OF ASPIRATED BONE MARROW

The volume of marrow which may be aspirated by puncture is limited, and the more material aspirated the greater is the proportion of contaminating blood; hence there is little if any advantage in aspirating more than 0.2 ml.

A well spread and well stained cellular film of marrow can be a delightful object, but the method of preparation of the film is as important as the staining technique. It is desirable to concentrate the marrow cells at the expense of the blood by which they are inevitably diluted.

The following simple device is generally satisfactory. A series of moderately large drops of syringe contents is delivered on to slides and most of the blood is then sucked off with a fine Pasteur pipette applied to one edge of each drop; the irregularly shaped marrow fragments tend to adhere to the slide and are left behind. A film is then made of the marrow fragments and the remaining blood by means of a smooth edged glass spreader of not more than 2 cm in width. The marrow fragments are dragged behind the spreader and leave a trail of cells behind them. It is in these cellular trails that differential counts should be done, commencing from the marrow fragment and working back towards the head of the film; in this way smaller numbers of cells from the peripheral blood become incorporated in the differential count.

Concentration of Marrow by Centrifugation. Several workers have used centrifugation techniques in an attempt to concentrate the marrow cells and to assess the relative distribution of marrow cells, peripheral blood and fat in aspirated material. Lamarzi (7), for instance, has devised a method for centrifuging heparinized aspirated marrow in a Wintrobe hæmatocrit tube "Yellow fat" "red fat," plasma marrow cells and mature ery- ... rrow cell layer

... llular samples

is useful, it seems unnecessary to do this to aspirated material of average or increased cellularity. While the volumetric data are undoubtedly of value in group studies, they are of less significance in individual cases because of the wide range of values encountered even in the normal

REPORTING ON PATHOLOGICAL MARROWS

Despite the difficulty of determining the limits of normality, examination of well-stained smears may give information of decisive diagnostic importance as well as providing information of great scientific interest; in no other way can disordered hæmopoiesis be so easily studied. A detailed "myelogram" is not often required in clinical practice. It is, however, useful to work out the leucocyte-erythroblast ratio by counting the number of nucleated red cells seen in a survey of, say, 500 nucleated cells. Although it may be masked by dilution with blood, a ratio of 2 to 1 or less may be certainly taken as indicating hyperplasia of the erythropoietic element. A record of this ratio and a report as to the type of erythropoiesis and the general maturity of the erythropoietic and leucopoietic cells is all that is usually needed when reporting on marrow smears for diagnostic purposes.

This is not to say that detailed differential counts are never useful and need never be done In leukæmia and in agranulocytosis, for instance, the proportion of primitive to maturing cells is important. As already mentioned, the best way to carry out the count is to start from a marrow fragment at the tail of the film and to count backwards towards the head of the film in the trail of cells which have been separated from the fragment during the spreading process.

On the other hand time is often much better spent in examining a series of slides than in blindly starting to perform a detailed differential count on the first few hundred cells seen; a wide search may reveal isolated groups of pathological cells, suggestive of metastatic carcinoma or diagnostic of a plasma cell tumour.

It should by now be unnecessary to stress the importance of a good technique of examining marrow films. To place a drop of oil on a film and to use the oil immersion objective straight away, is the worst possible thing to do. Films must first be inspected with the 2/3rd in. objective. Not until the best stained and most cellular area has been found, usually towards the tail of the film, should the oil immersion lens be used

Seaman also discuss the unavoidable statistical errors involved in performing differential counts, and the necessity for counting large numbers of cells particularly if an attempt is being made to assess the frequency of cells present in only small numbers. A further difficulty is the impossibility of accurately dividing into arbitrary classes cells of which every gradation of development may be seen.

For all the reasons referred to above and because of the naturally variegated pattern of the bone marrow and the irregular distribution of the marrow cells when spread in films, differential cell counts on marrow aspirated from normal subjects indicate a very wide range of normality, a range so wide that minor degrees of deviation from the normal occurring in disease are difficult to establish.

The normal values given in Table VII are based upon the data of several authors (8, 12–15). They can only be taken as an approximate guide.

Table VII

Normal Ranges for Differential Counts on Aspirated Bone Marrow, based on Various Authors (8, 12-15)

Reticulum cells	0–1%
Hæmocytoblasts	0–1%
Myeloblasts	0–2.0%
Promyelocytes	0 5–5.0%
Myelocytes	2–10%
Metamyelocytes (young forms) Metamyelocytes (stab forms)	5–30%
Neutrophil polymorphs	10–30%
Eosinophil myelocytes	0.1–2.0%
Eosinophils	0 1–2 0%
Basophils	0–0 5%
Lymphocytes	5–20%
Monocytes	0–2%
Megakaryocytes	0–0.5%
Plasma cells	0–1 5%
Pronormoblasts	0 5–2.0%
Normoblasts, basophilic	0 5–4.0%
Normoblasts, polychromatic	2–20%
Normoblasts, pyknotic*	2–10%
Erythroblast—leucocyte ratio	1 2 5–1.15

* The term "pyknotic" is preferred to "orthochromatic" as a description of the most mature normoblasts Cells with fully ripened cytoplasm (orthochromatic in the strict sense) are rarely met with in normal marrows

principles; how, for instance, cytoplasmic basophilia disappears as cells mature (except in plasma cells), that maturation is accompanied by nuclear condensation, that nucleoli are present only in relatively primitive cells, and that in leukæmia as in other forms of malignant disease, the cells encountered may not correspond in all particulars with their normal counterparts. Every hæmatologist should spend as much time as possible looking at films and should build up from the start his own atlas or museum by making a collection of slides, ideally each with its own index card on which essential particulars are recorded. Only well spread and well stained films should be kept—badly prepared ones are useless. He will ultimately find that he refers to his films far more often than he does to a text-book or atlas.

THE PREPARATION OF HISTOLOGICAL SECTIONS OF MARROW

The great advantage of sectioned material is that a picture of the marrow architecture as a whole is obtained. The proportion between cellular marrow and fat spaces is preserved, hypoplasia or hyperplasia can be recognized, and invasion by tumour or marrow fibrosis easily seen. Nevertheless, when it comes to cytological detail, sectioned material is much less satisfactory Not only are the subtle differences between cells such as normoblasts and megaloblasts which are easy to appreciate in stained films, difficult to recognize, but it may sometimes even be difficult to differentiate erythroblasts from leucocytes with complete certainty, particularly in decalcified postmortem material

It has already been mentioned that sections may be cut of the fragments of marrow aspirated by the puncture technique. These fragments are, however, small, rarely greater than 1.5 mm in size, and a careful technique in handling them is required. They are as a rule free from bone and the marrow architecture is well preserved, but their small size and the consequent uncertainty as to how representative of the marrow they are, limits their usefulness. A more serious disadvantage of the technique is that marrow frag-

A Note on the Recognition of the Different Types of Blood Cells

As has been mentioned in the preface to this work, no attempt is being made to describe or illustrate the various blood cells which may be met with in the bone marrow or in peripheral blood. This is primarily because such descriptions seem to be outside the scope of a book intended to be a guide to technique. There are, however, some other considerations.

Many Atlases of Hæmatology are extant; in some the illustrations are good, in others the reproductions are less satisfactory. It is clearly extremely difficult to reproduce on paper accurate pictures of blood cells. They rarely correspond *in all particulars with the objects with which you wish to* compare them. This is partly because all staining appearances are artefacts and the exact colours depend upon a whole sequence of factors; the thickness of the film, the age of the film, the adequacy of fixation, the stain itself, the proportion and reaction of the diluent, the length of time spent in staining, the length of time spent in differentiation—all these factors, and probably others too, influence the final result. It is difficult enough to get reproducible results in the same laboratory, let alone in different laboratories.

It is almost equally difficult to describe the blood cells as it is to illustrate them. Even cells indubitably of the same type vary substantially one from the other, and there is, moreover, a gradual gradation between the most primitive cells and the fully matured types which develop from them, and sometimes apparently even between types usually considered to be distinct, such as lymphocytes and monocytes, and promyelocytes and promonocytes. Any description to be worth while (i.e. useful in cases of difficulty) has to be detailed and comprehensive, if it is to be accurate, and thus inevitably long and cumbersome. Brief descriptions (or illustrations) of representative stages have only a limited value, and tend to over-simplify the problem.

Probably the best way for the beginner to learn to recognize the blood cells is to have the more important types demonstrated to him in films and then to try to comprehend what the processes of development involve and certain underlying

principles; how, for instance, cytoplasmic basophilia disappears as cells mature (except in plasma cells), that maturation is accompanied by nuclear condensation, that nucleoli are present only in relatively primitive cells, and that in leukæmia as in other forms of malignant disease, the cells encountered may not correspond in all particulars with their normal counterparts. Every hæmatologist should spend as much time as possible looking at films and should build up from the start his own atlas or museum by making a collection of slides, ideally each with its own index card on which essential particulars are recorded. Only well spread and well stained films should be kept—badly prepared ones are useless. He will ultimately find that he refers to his films far more often than he does to a text-book or atlas

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It has already been mentioned that sections may be cut of the fragments of marrow aspirated by the puncture technique. These fragments are, however, small, rarely greater than 1 5 mm. in size, and a careful technique in handling them is required They are as a rule free from bone and the marrow architecture is well preserved, but their small size and the consequent uncertainty as to how representative of the marrow they are, limits their usefulness A more serious disadvantage of the technique is that marrow frag-

ments are often not obtained by suction in just those patients with perhaps marrow aplasia, fibrosis or invasion by tumour, in whom histological evidence of any marrow abnormality is most urgently required. In these patients trephine biopsy may be necessary. A number of methods of dealing with aspirated fragments have been published (1) which differ in the details of handling the fragments, fixation and embedding. As with postmortem or trephine-biopsy material a mercury-containing fixative is required for good staining by Romanowsky dyes. For staining with hæmatoxylin and eosin, van Gieson or silver impregnation, formol saline suffices.

A Method for Staining Sectioned Material by May-Grünwald-Giemsa

The many different techniques which are in use for staining by Romanowsky dyes are evidence of the real difficulty in obtaining satisfactory results on sectioned material.

The following method* of staining with May-Grunwald-Giemsa stain gives quite good results. It may be applied to aspirated marrow fragments, trephine or postmortem material. If decalcification is necessary, the results are less satisfactory. In any case much depends upon fixation

Fix thin pieces of tissue as soon as possible in Helly's† fluid for 12–18 hours. Don't overfix. Wash in running water overnight. Place in Lugol's iodine for two minutes and then in 5 per cent sodium thiosulphate for two minutes Wash in distilled water and then rinse in distilled water buffered to *p*H 6 8

Stain for one hour in May-Grunwald stain diluted with an equal volume of buffered distilled water. Transfer without washing into Giemsa stain diluted with 19 volumes of buffered distilled water and leave for two hours By then the section will be grossly overstained and deep blue in colour

Differentiate by covering the section with a small volume of glycerin-ether (Gurr) freshly diluted with four parts of absolute ethyl alcohol Differentiation takes place quickly and is usually adequate in a few seconds Dehydrate by a rapid dip in absolute alcohol Clear in xylol and mount in Gurr's neutral mounting medium

The use of glycerin-ether helps to prevent "blueing" of the section during dehydration In a successfully stained section the

* I am indebted to Drs C V Harrison and A J M Reese for help with this method

† Helly's formol-sublimate solution consists of 5 per cent aqueous mercuric chloride to which 5 per cent by volume of formalin (40 per cent formaldehyde) is added just before use

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CHAPTER 7

LABORATORY METHODS USED IN THE INVESTIGATION OF THE HÆMOLYTIC ANÆMIAS

Spectroscopic examination of plasma; Schumm's test. Demonstration of "hæmosiderin" in urine. The [illegible] antibodies — "cold" hæmolysins, "cold" hæmagglutinins, "incomplete" antibodies and the Coombs test The differential agglutination method of studying the survival of transfused red cells The diagnosis of hæmolytic anæmia by laboratory methods

THE recognition of an increased rate of hæmolysis *in vivo depends very largely upon laboratory tests. These can be* divided into four categories; (1) the demonstration of an increased formation and excretion of normal or abnormal products of hæmoglobin metabolism, (2) the demonstration *in vitro* of an alteration in sensitivity of the patient's red cells to certain tests for hæmolysis, (3) tests for the presence or absence in the patient's serum or absorbed on to his cells of abnormal (hæmolytic) antibodies, and (4) the quantitative study of the survival *in vivo* of normal red cells transfused to patients.

Observations on the peripheral blood are naturally of great *importance also In a case of anæmia the presence of sphero-*cytes suggests that increased hæmolysis is taking place; so does a constantly increased percentage of reticulocytes, although it would be quite wrong to assume that because there is increased erythropoiesis, this is necessarily a response to hæmolysis, or conversely, that increased hæmolysis is necessarily accompanied by increased erythropoiesis. [illegible] and fragmenting corpuscles and Heinz bodies seen

in blood vitally stained with cresyl blue as for reticulocytes, suggest a chemically induced hæmolytic process.

EVIDENCE OF INCREASED HÆMOGLOBIN CATABOLISM

Neither the estimation of plasma bilirubin (1) nor the quantitative estimation of urobilinogen (2) will be described in this volume.* A brief note on the use of the spectroscope will, however, be given (3). In intravascular hæmolysis the characteristic absorption bands of oxyhæmoglobin in the green and yellow (at 540 and 578 mμ) may be seen in the plasma and in addition when hæmolysis has been severe or prolonged a faint band in the red (624 mμ) due to the presence of methæmalbumin

Schumm's Test. Small amounts of methæmalbumin can best be detected by the addition of approximately 1/10 part of ammonium sulphide to plasma or serum; a relatively intense band [illegible]

blood stream

In hæmoglobinuria, the oxyhæmoglobin in the urine may be accompanied by methæmoglobin The urine must be centrifuged to deposit red cells, if any should be present, before the supernatant is examined with a spectroscope.

Hæmosiderinuria. Where there is chronic hæmoglobinuria, as in nocturnal hæmoglobinuria (Marchiafava-Micheli disease) the urinary deposit may contain granules, in casts or in debris, which give a positive Prussian blue reaction for "free" iron.

Method The urine is centrifuged and the supernatant removed. It is replaced by an equal volume of a freshly made solution of 1 per cent potassium ferrocyanide in N/10 HCl (made by mixing

* [illegible]

[illegible]

equal volumes of 2 per cent potassium ferrocyanide and N/5 HCl). The deposit is resuspended in the acid-potassium ferrocyanide solution. After standing at room temperature for 5–10 min., the tube is centrifuged once more. The deposit is transferred to a slide, and covered with a cover glass. Often a blue colour is visible naked eye; if this is the case, very many isolated and grouped [illegible] aid of a microscope. [illegible] size as a rule. [illegible]" in the urine is prob- [illegible]

cells. In the absence of hæmolytic anæmia associated with hæmoglobinuria, "hæmosiderinuria" has been observed in both primary and post-transfusion hæmochromatosis (4).

TESTS OF CORPUSCULAR SENSITIVITY TO HÆMOLYSIS *IN VITRO*

The osmotic (hypotonic saline) fragility, mechanical fragility and "incubation" fragility (autohæmolysis) tests will be described.

The Osmotic Fragility Test

The estimation of corpuscular "fragility" has been widely used as an aid to the diagnosis of hæmolytic anæmia since the classic observations of Chauffard (5). It is, however, rather a difficult test to carry out with accuracy, and minor degrees of abnormality may be submerged in technical inaccuracies. Although extremely simple in principle, the result obtained depends not only on the cells themselves, but on *p*H, oxygen tension, temperature and the nature of the hypotonic solution and the proportion of blood added to it, as well as upon the care with which the test is carried out and the way the results are read.

Two techniques will be described, a simple technique in which hæmolysis is estimated visually, but which in careful hands is capable of giving consistent results, and a modification in which hæmolysis is read photoelectrically or photometrically.

A Simple Technique for the Estimation of Osmotic Fragility (6)

Required. A series of solutions of sodium chloride ranging from 0.85 to 0.10 per cent. These may be prepared in the following way :—

A stock solution of 10 per cent sodium chloride is made up as accurately as possible 25 g. of pure sodium chloride previously dried in a dessicator are transferred to a 250 ml. volumetric flask and the final volume made up to the mark with freshly distilled water after bringing to the required temperature This stock solution will keep indefinitely, from it a 1 per cent solution may be made The latter is used in the preparation of the range of hypotonic solutions. It is convenient to make 25 ml to 50 ml of each at a time, volumes of the salt solution and distilled water being delivered by burette, e g.

1% *NaCl*	*Distilled Water*	*NaCl concentration*
10.0 ml	15 0 ml	0 40%
12.5 ml.	12 5 ml.	0 50%
15.0 ml.	10 0 ml.	0 60%, etc

It is suggested that the following strengths should be prepared, or at any rate those marked with an asterisk (*)

0 85%*	0 75%	0 70%*	0 65%	0 60%*	0 55%
0 50%*	0 48%*	0 46%*	0 44%*	0 42%	0 40%*
0 35%*	0.30%*	0 25%	0 20%*	0 10%	

These solutions should be kept in tightly stoppered bottles in a cold room or refrigerator at a temperature of 1 to 4°C so as to restrain the growth of moulds If frozen, the solutions must be carefully mixed after the ice has melted A fresh series should be made up every 6 to 8 weeks

Method. Blood is withdrawn from a vein into a dry syringe and delivered into a tube containing heparin (0 2 mg. per ml. blood) On return to the laboratory this blood is thoroughly aerated by rotation in a tube or small conical flask for not less than two minutes until bright red. It is convenient to suck air over the surface of the blood whilst it is being rotated in a flask. The hypotonic salt solutions are then set up, 1 ml. volumes are delivered by pipette into a series of small tubes of equal bore. The exact range of solutions depends upon the requirements of the case, and whether for diagnostic or special purposes.

A Qualitative "Screening" Technique. The following strengths only need be used: 0.85, 0.48 and 0.30 per cent. One drop from a Dreyer pipette or 0.04 ml. of aerated blood is added to the solution in each tube and gently but thoroughly mixed with it. After standing at room temperature for thirty minutes, the three tubes should be centrifuged at 2000 rev. per minute for two minutes. In the normal case there should be no hæmolysis in the tube containing 0.48 per cent NaCl, and complete or practically complete hæmolysis with little or no macroscopic red button of unhæmolysed cells in the tube containing 0.30 per cent NaCl. The tube containing 0.85 per cent NaCl acts as a control. If there is any hæmolysis at all in 0.48 per cent NaCl, fragility is increased and the extent should be determined by the use of a wide range of salt solutions. Similarly, if increased resistance is denoted by a red button of cells in the tube containing 0.30 per cent NaCl, further tubes containing diminishing strengths of saline should be put up to determine the end point.

A More Accurate Quantitative Method. The packed cell volume of the blood to be tested should be adjusted to 45 per cent (the normal) after it has been aerated This standardizes the volume of plasma added with each volume of blood, and in anæmic patients facilitates estimation of hæmolysis 2 ml. of blood are delivered into a small tube, the cells being allowed to sediment whilst the packed cell volume of another sample is being estimated The amount of plasma to be withdrawn from the 2 ml. blood so as to adjust the packed cell volume to 45 per cent is calculated as follows —

Suppose P.C V = 30 per cent, then
2 ml. blood contain 0 60 ml cells

volume of plasma corresponding to 0 60 ml cells with P.C.V 45 per cent $= 0\ 60 \times \frac{55}{45} = 0\ 73$ ml

volume of plasma to be withdrawn from 2 ml sample
$= 2.0 - (0\ 60 + 0\ 73)$ ml
$= 2.0 - 1\ 33$ ml
$= 0\ 67$ ml.

0.04 ml. blood is added to 1 ml. of a range of saline solutions using a capillary automatic or Dreyer pipette. The range of saline to be used is based on the results of a preliminary qualitative estimation. In addition 3 × 0 04 ml. volumes of blood

are added to 3 ml. of distilled water. After 30 minutes at room temperature the tubes are centrifuged. The amount of hæmolysis can then be read by direct matching with standards made from the blood hæmolysed in distilled water ("100 per cent" hæmolysis). By dilution, tubes corresponding to 2½, 5, 10, 20, 30, 40, 50, 60, 70 and 80 per cent are prepared, e g

Distilled water	0.1	0.15	0.2	0.25	0.3	0.35	0.4	0.85	0.9	ml.,
Hæmolysed blood	0.4	0.35	0.3	0.25	0.2	0.15	0.1	0.15	0.1	ml.
Lysis	80%	70%	60%	50%	40%	30%	20%	15%	10%	

(2½ and 5 per cent concentrations are prepared from the 10 per cent tube.)

It is difficult to estimate differences in hæmolysis between 80 and 100 per cent; in most instances, fortunately, this is of no consequence Amounts of hæmolysis smaller than 2½ per cent can be readily detected naked eye and should be recorded as "traces." Such "traces" are significant, but the tubes should always be compared with the tube containing 0.85 per cent saline which acts as a control The temperature of the hypotonic solution affects the amount of hæmolysis—the lower the temperature the greater the hæmolysis in any salt concentration For increased accuracy the tubes may be immersed in crushed ice at 0°C or in a water bath at 20°C , in practice, room temperature is usually sufficiently constant

Normal Limits (6)

"Initial" hæmolysis	0.42–0.46% NaCl
M.C.F (50% hæmolysis)	0.34–0.40% NaCl
"Complete" (99%) hæmolysis	0.28–0.32% NaCl

Determination of Osmotic Fragility by a Photoelectric Method

The technique previously described has certain disadvantages; the method of adding drops of blood to saline, although convenient, is inaccurate, as is also the visual method of reading the percentage of hæmolysis, where hæmolysis is nearly complete The complete procedure is time-consuming. It is, however, capable of giving closely reproducible results

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Suppose P.C.V. = 30 per cent, then
2 ml. blood contain 0.60 ml. cells.

volume of plasma corresponding to 0.60 ml. cells with P.C.V.

45 per cent $= 0.60 \times \frac{55}{45} = 0.73$ ml.

∴ volume of plasma to be withdrawn from 2 ml. sample
$= 2.0 - (0.60 + 0.73)$ ml.
$= 2.0 - 1.33$ ml.
$= 0.67$ ml.

0.04 ml. blood is added to 1 ml. of a range of saline solutions using a capillary automatic or Dreyer pipette. The range of saline to be used is based on the results of a preliminary qualitative estimation. In addition 3 × 0.04 ml. volumes of blood

100 per cent hæmolysis which is impossible by naked-eye matching.

As one part of blood is added to 100 parts of saline, the osmotic equivalence of the added plasma is extremely small and can be neglected. The effect of anæmia is similarly negligible. The temperature should be a constant one; 20°C. is convenient.

Buffered saline is required in this technique because the small amount of plasma added is less effective in producing a constant *p*H than the larger amounts (×4) added in the method previously described. Despite the use of buffered saline, however, fragility is slightly affected by the proportion of oxyhæmoglobin to reduced hæmoglobin. To achieve standard conditions, the blood must be maximally aerated.

Normal Range (20°C.)

0.30% NaCl	97–100%	hæmolysis
0 35% ,,	90–99%	,,
0.40% ,,	50–90%	,,
0 45% ,,	5–30%	,,
0.50% ,,	0–5%	,,
0.55% ,,	nil	,,

The Significance of Osmotic Fragility Tests

As has already been indicated, many factors affect the results of the osmotic fragility test, and for this reason the "normal range" of fragility has to be worked out for each method For the factors which control the hæmolysis of corpuscles in hypotonic solutions and for the complex theoretical background, and for information as to how the behaviour of pathological corpuscles may differ from the normal, the reader is referred to Ponder's monograph (8) and to a recent paper by Guest (9) It is generally agreed that the more nearly spheroidal a cell, the less it can swell in a hypotonic medium without stretching the cell membrane. "Spherocytes" are hæmolysed in solutions which are insufficiently hypotonic to hæmolyse normal more discoidal cells of the same volume as the spherocytes but with larger

Nevertheless, the addition of varying amounts of plasma along with the corpuscles, according to the degree of anæmia present, leads to inaccuracy, and the final pH depends on the efficiency of aeration.

In the following method, based on that of Parpart *et al.* (7), some of these objections are overcome.

Required. A stock solution of sodium chloride buffered with phosphates to pH 7.4, osmotically equivalent to 10 per cent sodium chloride. This is prepared as follows: NaCl 180 g., Na_2HPO_4 27.31 g. (anhydrous weight) and NaH_2PO_4 3.74 g. (anhydrous weight) are dissolved in distilled water and the final volume adjusted to two litres. For use, this stock solution is diluted with nine times its volume of distilled water, making it osmotically equivalent to 1 per cent sodium chloride. This latter solution is diluted with distilled water, as described for the previous technique, so as to make a range of hypotonic solutions. Dilutions equivalent to 0.85, 0.75, 0.65, 0.60, 0.55, 0.50, 0.45, 0.40, 0.35, 0.30, 0.20, and 0.10 per cent NaCl are recommended. These solutions may be kept at 1–4°C. in stoppered bottles, but must be discarded if moulds grow,

Method Heparinized venous blood aerated as previously described or fresh capillary blood may be used. 0.05 ml volumes are washed into and well mixed with 5 ml. volumes of a suitable range of hypotonic solutions Additional 0.05 ml. volumes are added to 5 ml. N/200 NaOH and 5 ml. 0.85 per cent sodium chloride, which serve as controls.

After thirty minutes at 20°C. the tubes are centrifuged and the supernatant liberated hæmoglobin estimated in a photo-electric colorimeter or photometer, using a green filter.

The blood added to the tube containing N/200 NaOH is completely hæmolysed and is equivalent to 100 per cent hæmolysis; the supernatant from the tube equivalent to 0.85 per cent sodium chloride is the blank representing no hæmolysis

Minor degrees of hæmolysis (less than 2 per cent) are not satisfactorily estimated with ordinary equipment. They can be observed by eye, however, and may be recorded as "traces." The method can, however, easily distinguish between 95 and

A Note on Recording the Results of the Osmotic Fragility Test. Most authors who have estimated osmotic fragility quantitatively have plotted the totals of hæmolysis in each tube against the corresponding concentrations of salt solution, obtaining in this way in normal subjects a curve of sigmoid shape (see Fig. 7). In disease, deviations from the normal form are found ; the curves may for instance have long "tails" due to the presence of a small proportion of very fragile cells, or types of curves intermediate between this type and the normal may be found (10) (see Fig. 7). Alternatively, "increment hæmolysis curves" can be drawn

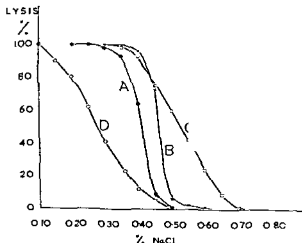


FIG 7 NORMAL AND ABNORMAL OSMOTIC FRAGILITY CURVES
(Modified method of Parpart *et al*)

A. [illegible]
B
C
D [illegible] fibro-congestive splenomegaly

(11, 12) , in this case the differences in hæmolysis between adjacent tubes are plotted against saline concentrations. Ponder (13) plots the differential of the percentage hæmolysis curve against the saline concentration. These methods well demonstrate the presence or absence of homogeneity in the

surface areas. A significant increase in osmotic fragility* is almost diagnostic of a hæmolytic anæmia ; on the other hand, decreased osmotic fragility (increased resistance) is found in iron deficiency anæmias and particularly in Mediterranean anæmia, chronic liver disease and after splenectomy. However, an increase in osmotic fragility is *not* diagnostic of any particular type of hæmolytic anæmia ; a variable increase is found in many, but not in all, hæmolytic anæmias of acquired type as well as in the classical familial type (see Fig. 6).

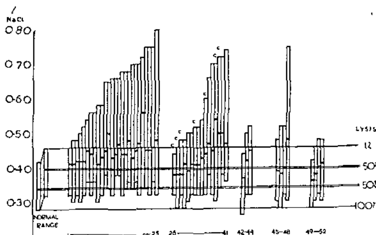


Fig. 6 The Results of Osmotic Fragility Tests in 52 Cases of Congenital or Acquired Hæmolytic Anæmia

(Modified Dacie and Vaughan's method)

Patients 1–25 were examples of familial hæmolytic anæmia (congenital acholuric jaundice)—all the tests were abnormal Patients 26–41 were examples of idiopathic acquired hæmolytic anæmia—in two patients the fragility was within the normal range C indicates that a Coombs test was carried out and found to be positive Patients 42–44 were examples of secondary hæmolytic anæmia—in one case the fragility was normal Patients 45–48 were examples of chemical hæmolytic anæmia—in each case the fragility was abnormal
—in twc
indicate
lysis (M
fragility

* Increased osmotic fragility has been observed in myelosclerosis and in carcinomatosis of bones, in both disorders, however, there may be a hæmolytic element to the anæmia

The test should be carried out as follows :—

Incubation of Clotted Blood. Blood should be collected from a vein and carefully delivered in 1 ml. volumes without frothing into several sterile 80×10 mm. tubes. The blood is left undisturbed and allowed to clot at room temperature. When the clots have started to retract and the loose cells by sedimenting have revealed clear yellow serum, one tube is kept in the refrigerator to act as a control and the others placed in a water bath at 37°C. and left there for 2–3 hours If autohæmolysis is taking place, red hæmoglobin pigment will diffuse from the clot into the clear serum in the tubes kept at 37°C., but not in the control. This procedure has been described as a heat resistance test (16) ; the choice of name seems an unfortunate one as the blood is not subjected to heat greater than body temperature.

This test is positive within two hours in nocturnal hæmoglobinuria and in rare instances of idiopathic acquired hæmolytic

sterile blood, 2–5 ml in volume, are delivered into small sterile screw-capped bottles After incubation for 24–48 hours the contents of one or more bottles are well mixed and then centrifuged Volumes of the supernatants are then diluted in N/10 NaOH so that the liberated hæmoglobin may be estimated as alkaline hæmatin. Standards are prepared in a similar way from whole blood and from serum separated off before incubation The results of applying this procedure to a range of normal and pathological bloods are illustrated in Fig 8.

Test for Hæmolysis in Acidified Serum [Ham's Test (37)]

This technique is particularly used in the diagnosis of nocturnal hæmoglobinuria. In this disorder the patient's corpuscles are sensitive to thermolabile components of normal serum The purpose of acidification is to adjust the *p*H of the corpuscle-serum suspension to the optimum for the action of the serum factors (about *p*H 6.8) This is necessary because when serum is obtained by defibrination or after blood has clotted, it becomes slightly alkaline (*p*H 8 0) due to loss of carbon dioxide. This sensitivity to changes in

population of corpuscles; definitely bimodal curves may be obtained during recovery from a hæmolytic episode.

Mechanical Fragility

Normal red cells are susceptible to mechanical trauma and may be readily hæmolysed *in vitro* by shaking with glass beads. Increased sensitivity has been observed in certain pathological states, and the test if performed quantitatively can be used in the investigation of hæmolytic disorders (14). Spherocytes, incubated, and agglutinated corpuscles and sickled cells have an increased sensitivity to mechanical traumata, and this is probably also true of poikilocytes.

It is possible, but difficult to prove, that mechanical trauma may play an important role in hæmolysis *in vivo*, both in health and in disease.

The mechanical shaking test has to be standardized for each laboratory. Amongst the factors which need to be considered, are the volume of blood, its packed cell volume which needs to be adjusted to a constant value, the size of the container, the number and size of the beads added to the blood, the temperature at which the blood is shaken, the speed and amplitude of the shaking device and the time for which the blood is shaken.

The test is unlikely to be used in clinical diagnosis, but it is a useful research method.

Incubation Fragility (Autohæmolysis)

A simple method of investigation of a suspected case of hæmolytic anæmia is to observe the effect of incubating at 37°C. for 24–48 hours sterile samples of clotted or defibrinated blood (15). Accelerated hæmolysis will be observed in a variety of conditions, in familial hæmolytic anæmia (acholuric jaundice), in idiopathic acquired hæmolytic anæmia, in hæmolytic anæmias due to drugs or chemicals, in "cold" hæmoglobinuria if the sample is chilled before incubation, and particularly in nocturnal hæmoglobinuria. The mechanism of accelerated hæmolysis differs from case to case, and a positive test should be looked upon solely as a pointer to a hæmolytic process

not complete, hæmolysis of patient's corpuscles in acidified normal serum, and absent or a trace of hæmolysis in unacidified normal serum. The tubes containing the suspension of patient's corpuscles in heated acidified serum and the suspension of normal corpuscles in acidified normal serum should be free from hæmolysis.

THE DEMONSTRATION OF ABNORMAL AUTO-ANTIBODIES IN HÆMOLYTIC ANÆMIA

It is now recognized that some cases of hæmolytic anæmia are due to the spontaneous development of anti-red cell antibodies. The demonstration of the presence or absence of these antibodies is thus a necessary and important step in the investigation of any patient suffering from a hæmolytic anæmia. Although auto-antibody "free" in the patient's serum may be difficult to demonstrate, antibody absorbed on to the surface of the patient's corpuscles is quite frequently found (19). In the following sections are described methods for the demonstration of serum hæmolysins of the "cold" (Donath-Landsteiner) type and of the "warm" type, and methods for the detection and titration of "cold" hæmagglutinins and "incomplete" antibodies in the serum, and "incomplete" antibodies absorbed on to corpuscles

Tests for Serum Hæmolysins of the "Cold" Type

The principle of the Donath-Landsteiner test (20) is the exposure of red cells suspended in the patient's serum first to a low temperature so that the "cold" hæmolysin may be absorbed, and then to a higher temperature so that hæmolysis may be completed by the action of serum complement. The test may be carried out in two ways:—

(1) The patient's blood is withdrawn, allowed to clot and inspected for the subsequent presence or absence of spontaneous hæmolysis. Venous blood should be delivered into several previously warmed tubes and allowed to clot at 37°C. When the clot has commenced to retract and clear yellow serum is visible, one or more tubes are placed in a refrigerator at 2–5°C. for 15–30 minutes leaving a control tube in the water bath at 37°C. The chilled tubes are then replaced in the water bath at 37°C. and inspected at intervals for the onset of hæmolysis; with an active

pH is characteristic of the reaction in nocturnal hæmoglobinuria, but operates in other hæmolytic reactions also (17).

Method. An approximately 50 per cent suspension is made from patient's corpuscles washed in saline. Fresh normal compatible adult serum is acidified with 1/10 of its volume of N/5 HCl. To the acidified serum 1/10 volume of the 50 per cent suspension of patient's cells is then added; i.e. serum 1.0 *ml*, N/5 HCl, 0.1 ml., 50 per cent cell suspension 0.1 ml, added after the acid has been carefully mixed with the serum. The final *pH* of the suspension is about 6.8.* It is important not to dilute the serum unduly; hence small volumes of a relatively strong acid and cell suspension are used. As controls, further suspensions using

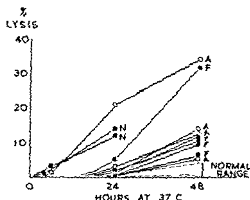


FIG. 8. ACCELERATED HÆMOLYSIS OF DEFIBRINATED BLOOD FROM CASES OF HÆMOLYTIC ANÆMIA WHEN INCUBATED UNDER STERILE CONDITIONS AT 37°C.

A. Cases of idiopathic acquired hæmolytic anæmia
F. Cases of familial hæmolytic anæmia
N. Cases of nocturnal hæmoglobinuria

unacidified serum and patient's corpuscles, acidified serum and normal corpuscles, and inactivated† acidified serum and patient's corpuscles should be set up.

The suspensions are incubated at 37°C for 30–60 minutes. The tubes are then centrifuged and the supernatants examined for hæmolysis. In nocturnal hæmoglobinuria there will be definite, but

* By varying the strength of the acid used (N/20 to N/2.5) *pH*-lysis curves may be obtained; in nocturnal hæmoglobinuria the range of *pH* between which hæmolysis can be demonstrated is from 8 to 6 approximately (18).

† Heated to 56°C. for 15 minutes before acidification.

of the Donath-Landsteiner reaction are given in recent papers by Siebens, Zinkham and Wagley (21) and Dacie (39).

Tests for Serum Hæmolysins of the "Warm" Type

In rare cases of acute hæmolytic anæmia, pan-hæmolysins have been demonstrated in the patient's serum (22, 23)

throughout. It is likely that in some cases the absorption of the abnormal hæmolysin is affected by the pH of the system, the hæmolysin being best absorbed to the acid side of the pH range 8–6 (22) No tests for hæmolysins should, therefore, be considered negative until the following procedure has been carried out:—

Normal group O corpuscles are sensitized in the patient's acidified serum by adding to 0 5 ml of the patient's serum, 0 05 ml N/3 HCl and then 0 05 ml. of a 50 per cent suspension of washed normal corpuscles The pH of the suspension will be approximately 6 2. After 30 minutes incubation at 37°C, the

be present

ı is present

the serum.

If there is no hæmolysis, the suspension should be centrifuged, the supernatant removed by pipette and 0 5ml. of fresh normal compatible serum acidified with 0 05 ml N/4 5 HCl added to the deposit (the pH of the serum should be 6 6–6 8, about optimal for the action of complement). The corpuscles are resuspended and the suspension is incubated for a further one hour at 37°C It is then centrifuged and examined for hæmolysis As a control the experiment should be duplicated using normal serum throughout

Detection and Titration of Abnormal Autohæmagglutinins

The phenomenon of autohæmagglutination is commonly observed in hæmolytic anæmias of the idiopathic acquired type (23, 24).* In most cases the antibody is of the "cold" type, although usually of sufficiently high titre and thermal amplitude for agglutination to take place at room temperature.

hæmolysin thus should be visible within a few minutes. The blood in the control tube kept at 37°C. throughout the experiment should not undergo hæmolysis. Although 37°C. is the conventional temperature employed in the second stage (warm phase) of the Donath-Landsteiner reaction, it is likely that a temperature lower than this is really the optimum. Although a temperature of 37°C. may be almost optimal for the action of serum complement, at this temperature antibody is very rapidly shed from the corpuscles. At lower temperatures, although complement is less active, elution of antibody takes place more slowly, and thus may more than compensate for the reduced activity of the complement (39).

(2) Patient's blood is allowed to clot at 37°C. and the serum separated at this temperature. Normal washed corpuscles are used to test for the presence of "cold" hæmolysin in the serum. To 10 vol. of fresh patient's serum is added 1 vol. of a 50 per cent suspension of normal compatible or group O washed corpuscles. The red cells are sensitized at 2–5°C. for 15–30 minutes. The suspension is then warmed to 37°C. Hæmolysis will take place within a few minutes if the hæmolysin is active. A control tube containing 10 vol. of warmed patient's serum to which has been added 1 vol. of warmed cell suspension should be set up at the same time and kept at 37°C. throughout. No hæmolysis should be observed.

With very active hæmolysins, chilling at temperatures as low as 2–5°C. may not be necessary and cooling to room temperature (15–20°C.) may be sufficient. In the case of stored serum, fresh serum complement should be added to the patient's serum, either a half-volume of fresh human serum or a 1/10 volume of fresh guinea pig serum, or after sensitizing the cells in the cold in the patient's serum, the suspension may be centrifuged and the patient's serum replaced by fresh normal serum before warming. The patient's serum should not be inactivated by heat, for some hæmolysins appear to be destroyed if the serum is heated to 56°C. (21). In other cases, although the hæmolysin is not thermolabile, its absorption may be inhibited if the serum is heated (39).

A patient's serum hæmolysin may be titrated by making falling halving dilutions in saline and adding equal volumes of serum complement to each tube. Equal volumes of cell suspension are added and the tubes chilled and warmed as before.

It is probable that if unacidified serum is used the *p*H of the system will not be optimal. Nevertheless it is generally unnecessary to make any adjustment of *p*H, although by acidifying the serum with 1/10 of its volume of N/5 HCl the amount of hæmolysis can be increased* (22). Other details as to the complexity

* It has been recently demonstrated that in the case of certain cold hæmolysins found to be present in sera containing cold hæmagglutinins at high titres, hæmolysis may not take place unless the serum has been acidified (39).

of the Donath-Landsteiner reaction are given in recent papers by Siebens, Zinkham and Wagley (21) and Dacie (39).

Tests for Serum Hæmolysins of the "Warm" Type

abnormal hæmolysin is affected by the pH of the system, the hæmolysin being best absorbed to the acid side of the pH range 8–6 (22). No tests for hæmolysins should, therefore, be considered negative until the following procedure has been carried out:—

Normal group O corpuscles are sensitized in the patient's acidified serum by adding to 0.5 ml of the patient's serum, 0.05 ml N/3 HCl and then 0.05 ml. of a 50 per cent suspension of washed normal corpuscles. The pH of the suspension will be *approximately 6.2. After 30 minutes incubation at 37°C. the*

supernatant removed by pipette and 0.5ml. of fresh normal compatible serum acidified with 0.05 ml. N/4.5 HCl added to the deposit (the pH of the serum should be 6.6–6.8, about optimal for the action of complement). The corpuscles are resuspended and the suspension is incubated for a further one hour at 37°C. It is then centrifuged and examined for hæmolysis. As a control the experiment should be duplicated using normal serum throughout.

Detection and Titration of Abnormal Autohæmagglutinins

The phenomenon of autohæmagglutination is commonly observed in hæmolytic anæmias of the idiopathic acquired type (23, 24).* In most cases the antibody is of the "cold" type, although usually of sufficiently high titre and thermal *amplitude for agglutination to take place at room temperature.*

Rarely agglutination may persist even at body temperature (25); in these cases hæmolysis *in vivo* is usually extremely serious.

"Cold" agglutination may be observed by inspection of whole defibrinated blood or blood to which an anticoagulant has been added The rapid spontaneous development of marked granularity after withdrawal is suggestive, and the presence of autohæmagglutinins is confirmed if the granularity increases when the sample is cooled in a refrigerator. These agglutinins seem always to be "panhæmagglutinins" and affect both normal and patient's corpuscles similarly. In titrations it is convenient to use suspensions of normal corpuscles.

"Cold" agglutinins may be titrated in the following way:—

The patient's blood is allowed to clot at 37°C. When the clot has retracted, serum is removed by pipette and freed from any suspended cells by rapidly centrifuging whilst still warm. Falling halving dilutions of this serum are then made in normal saline. Usually a dilution range from 1:2 to 1:2048 is sufficient, occasionally the range needs extending. To each tube is then added an equal volume of a 1 per cent suspension of washed normal group O corpuscles. The rack of tubes is gently shaken and allowed to rest undisturbed at room temperature for two hours

At the end of this time the end point of any agglutination taking place at room temperature is read off This can be done by gently inverting the tubes two or three times and examining the suspensions of corpuscles with the aid of a concave mirror, or by placing small drops of the suspensions on slides and examining them under the microscope. The microscopic end point is taken as the greatest dilution in which clumps of four to five cells or more are evenly distributed amongst many unagglutinated corpuscles The concave-mirror method is about as sensitive as is the microscopic-end point method. In every case careful comparison must be made with the control tube in which the corpuscles are suspended in saline alone

Inspection of the pattern of the deposit will often indicate most sensitively the presence or absence of agglutination—a

roughness or irregularity of the deposited corpuscles in place of a perfectly smooth button indicating agglutination. Results read in this way should, however, be checked by inspection after the deposited cells have been resuspended.

When the results of standing at room temperature have been read, the deposited corpuscles are resuspended and the rack of tubes is placed in a refrigerator at 2–5°C. and left undisturbed for a further two hours, or, if more convenient, overnight. The titre of agglutination is then redetermined before the tubes have had time to warm up. Finally, the corpuscles are resuspended once more and the rack placed in a water bath at 37°C. for one hour and the presence or absence of agglutination determined once again.

The following are typical results :—

Agglutination titre* at 2–5°C.	1024
Agglutination titre at 17°C.	64
Agglutination titre at 37°C.	nil

Detection of "Incomplete" Antibodies

Incomplete (28) or blocking (29) or (in) albumin-agglutinating (30) antibodies were first described in connection with Rh iso-immunization and hæmolytic disease of the newborn. The nature of these antibodies is such that agglutination will generally not take place except in a medium of high protein concentration, and thus their presence will not be detected by ordinary methods if saline is used as a medium for suspending the red cells or as a diluent for serum.

The importance of antibodies of a similar type in cases of idiopathic acquired hæmolytic anæmia has been recently recognized (19) In these patients it will be found that corpuscles of all blood groups as well as the patients' own red cells will be sensitive to them. The presence of antibodies of this type is most reliably detected by carrying out a "direct" Coombs test upon the patient's own corpuscles.

Detection of Absorbed Antibody by the "Direct" Coombs Test (Anti-globulin Test). The patient's red cells are well washed free from plasma in several changes of a

* Expressed as the reciprocal of the final serum dilution

large volume of saline. An approximate 5 per cent suspension of the washed corpuscles is then made and two large drops placed upon an opalescent white tile or slide. To one drop is added a drop of the anti-globulin rabbit serum* and to the other a drop of saline; the latter acts as a control suspension. The tile or slide is gently rocked and warmed and inspected for the development of agglutination. In a positive reaction agglutination develops within ten minutes, in strong reactions within a matter of seconds perhaps. The control suspension should show no agglutination. Doubtful reactions should be confirmed microscopically. This is a most sensitive test, but its sensitivity depends greatly upon the potency of the anti-globulin serum as well as upon the degree of sensitization of the corpuscles. Control suspensions using corpuscles deliberately sensitized with a weak anti-Rh incomplete antibody (see later—detection of Rh antibodies) and normal unsensitized cells should always be put up at the same time to test for the efficacy of the anti-globulin serum.

Detection of Incomplete Antibody in the Patient's Serum. Incomplete antibodies have not often been detected in the sera of patients with idiopathic acquired hæmolytic anæmia (31). More frequently they are found in the cord serum of babies affected with hæmolytic disease of the newborn. Possibly in both cases success or failure depends greatly on the sensitivity of the methods used for their detection. Methods for the detection of Rh incomplete antibodies are described in the next chapter. In acquired hæmolytic anæmia in adults the same procedure should be used but in this case it is well to use group O Rh negative corpuscles as the test corpuscles to avoid as far as possible the complicating effects of iso-antibodies. The corpuscles are suspended in the suspected serum for 1–2 hours at 37° C. so that they may become sensitized. A Coombs test is then performed on the corpuscles after they have been thoroughly washed in saline ("indirect" Coombs test). It is essential to carry out the sensitization at 37°C. "Cold" incomplete antibodies exist in many normal sera. False positive results may be obtained if the tests are performed at room temperature. Alternatively, a suspension of corpuscles in 20 per cent bovine albumin may be added to the serum, or the corpuscles may be treated with trypsin (32) before being added to the serum under test. Details of these methods are given in Chapter 9.

* The method of preparation of this serum is described in Chapter 9.

THE STUDY OF THE SURVIVAL OF TRANSFUSED RED CELLS BY THE DIFFERENTIAL AGGLUTINATION METHOD OF ASHBY (33)

The investigation of patients with hæmolytic anæmia has been greatly facilitated by quantitative studies of the survival of transfused normal corpuscles. In this way it has been possible to divide cases of hæmolytic anæmia into two broad groups; those in which normal corpuscles survive in the patient for the normal length of time (about 120 days), as in familial hæmolytic anæmia (33), nocturnal hæmoglobinuria (34, 36) and sickle cell anæmia (35), and those cases of varying pathogenesis in which the rate of destruction of transfused cells is increased (36). In the first group the increased rate of hæmolysis results from some abnormality of the patient's own corpuscles; in the second group the hæmolytic mechanism is one which treats both the patient's corpuscles and normal corpuscles alike.

The Principle of the Ashby Method. The general idea is to transfuse into a recipient corpuscles of a different, but compatible, blood group, e g. the transfusion of O red cells into A, B or AB, ON red cells into OM or OMN, or ORh — red cells into ORh + recipients, etc. The resulting mixture of corpuscles may be separated if they are suspended in an agglutinating serum to which the recipient's corpuscles are sensitive, but not those of the donor; e g. the recipient's A corpuscles would be clumped by anti-A serum, but the donor's O corpuscles would not be affected and would remain in a dispersed suspension. Moreover, if a known but small quantity of this blood containing both O and A cells is suspended in a relatively large volume of serum so as to make a 1.100 or 1:200 dilution, the actual number of unagglutinated corpuscles may be counted as in an ordinary blood count.

The avidity and titre of the serum is of the highest importance; the titre should be 1000 or more. As anti-Rh sera of this titre are rare, and anti-M or N sera in any case difficult to prepare, the investigator is usually restricted to the use of

anti-A and anti-B, and thus to tracing the survival of O blood transfused into A, B or AB recipients.*

Technique. 0.1 ml of blood is suspended in 4.9 ml. of 3 per cent sodium citrate solution so as to make a 1 in 50 dilution. The blood may be venous in origin or from a freely bleeding skin prick. One volume of the suspension is then added to one volume of the appropriate agglutinating serum in a 80×10 mm. tube provided with a cork; 0.25 ml. volumes are suitable.

It is good practice to do the test in duplicate if sufficient serum is available. The serum should be used undiluted or diluted in several volumes of saline, if necessary, to the concentration at which agglutination is maximal, as shown by preliminary experiments with the recipient's blood. Agglutination of the recipient's cells should be intense before transfusion and there should be very few free cells if a good serum is used, the best results will be obtained if there are less than 20,000 free cells per c.mm. when blood containing 5.0 million red cells per c mm. is agglutinated by the technique to be described.

The dilution of blood in citrate and serum (now 1 in 100) is left at room temperature for at least one hour, and then centrifuged at about 1500 rev. per minute for one minute. The tubes are then quite vigorously shaken so that not only are the unagglutinated cells suspended, but the button of agglutinated cells becomes broken up into small but still visible fragments. After waiting for not more than one minute, during which time the largest clumps of agglutinated cells will sink to the bottom of the tube, the upper three-quarters of the suspension consisting of free cells and small clumps only, is removed by Pasteur pipette into a fresh tube. This tube is corked and the contents centrifuged for one minute as before. The button of deposited cells is then well mixed with the supernatant fluid by a standard procedure—fifty inversions through an angle of 90° to 120° at the rate of one per second. A counting chamber is then filled from the

* Care must be taken that the plasma of the transfused group O blood does not contain anti-A or anti-B at high titres. Dangerous reactions due to hæmolysis of the recipient's corpuscles may result from this (see Chapter 9).

upper layers of the cell suspension thus minimizing the number of agglutinates withdrawn.

After waiting about three minutes for the cells to settle, a red cell count is performed in the usual way, counting, however, "free" cells only; the cells (usually tightly agglutinated) in the few clumps which may be seen are ignored. The number of unagglutinable (donor) cells present may be expressed in absolute numbers or as a percentage of the number present at the conclusion of the transfusion.

THE DIAGNOSIS OF HÆMOLYTIC ANÆMIA BY LABORATORY METHODS

In the following scheme is summarized a suggested order for carrying out laboratory investigations on a patient suspected of suffering from hæmolytic anæmia.

1. Estimation of hæmoglobin and inspection of a stained blood film. Often a tentative diagnosis can be made at this stage, by noting the presence of spherocytes and polychromasia
2. A reticulocyte count; this will confirm or refute what has been suspected by examination of the stained film.
3. If the patient is anæmic, a red cell count, hæmoglobin and packed cell volume estimation carried out on venous blood.
4. Plasma bilirubin estimation and if indicated spectroscopic examination of the patient's plasma; also examination of the urine for abnormal pigments
5. Special tests applicable to cases of hæmolytic anæmia, and which may help to define the mechanism of the increased hæmolysis (outlined in this Chapter)
6. Bone marrow biopsy. This will give an idea of the intensity of erythropoiesis
7. Study of survival of transfused corpuscles and/or quantitative estimation of fæcal urobilinogen

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Gray, C H, and Whidb[illegible]
den Bergh reaction" *Bio* [illegible]

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5. Special tests applicable to cases of hæmolytic anæmia, and which may help to define the mechanism of the increased hæmolysis (outlined in this Chapter).
6. Bone marrow biopsy. This will give an idea of the intensity of erythropoiesis.
7. Study of survival of transfused corpuscles and/or quantitative estimation of fæcal urobilinogen

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LABORATORY METHODS USED IN THE *INVESTIGATION OF PATIENTS SUFFERING* FROM HÆMORRHAGIC DISORDERS

The platelet count. The coagulation time. The bleeding time. Estimation of prothrombin time. Quantitative measurement of clot retraction. The diagnosis of the cause of a hæmorrhagic tendency.

A LIABILITY to spontaneous hæmorrhage or an increased tendency to bleed following trauma or surgical incisions may result from a wide range of disorders. The proper investigation of patients suspected of suffering from a hæmorrhagic disease is both difficult and troublesome, and the results obtained are usually more inexact and unreliable than in any other branch of hæmatology. While it is true that the investigation and elucidation of an obscure tendency to bleed needs special knowledge and experience, and special reagents, there are certain routine methods which if carried out efficiently can give results of diagnostic importance These procedures are the platelet count, the coagulation time, the one-stage "prothrombin time" and the bleeding time. A method for measuring clot retraction will also be described although this technique is less likely to yield results of practical importance.

THE PLATELET COUNT

Many methods for the enumeration of the blood platelets have been published. Their number is doubtless due to the real difficulty of counting small bodies which agglutinate and break up so easily, and which may be difficult to separate from extraneous matter. No attempt will be made to review recommended methods in detail, the reader is referred to Tocantins' (1, 2) excellent reviews for technical and other

details concerning mammalian platelets. In the present chapter two simple techniques only will be described; Lempert's modification of Kristenson's method (3); a "direct" method of counting the platelets in peripheral blood; and an "indirect" method suitable for use with venous blood.

Lempert's Modification of Kristenson's Platelet Counting Technique

Two solutions are required :—

Solution A.	Sodium citrate	1.0 g.
	Mercuric chloride	0.002 g.
	Brilliant cresyl blue	0.2 g.

The reagents are dissolved in 100 ml. distilled water at 45°C.

Solution B.	Urea	20.0 g
	Distilled water to 100 ml.	

Equal volumes of solutions A and B are mixed when required. The mixture will keep for several days but must be filtered or centrifuged before use The patient's ear is warmed by rubbing with lint. The mixed stain is drawn up in a leucocyte counting pipette to the 0 5 mark The patient's ear is pricked sufficiently deeply so as to obtain a free flow of blood. Blood is sucked up to the 1.0 mark, and then the pipette is filled with stain up to the 11 mark The pipette is shaken for one minute. On returning to the laboratory the contents of the pipette are remixed for two minutes and a counting chamber filled This is placed in a Petrie dish containing a pledget of damp cotton wool and allowed to stand for twenty minutes to one hour This interval is necessary to ensure sedimentation of the platelets and so that the red cells may be hæmolysed.

The platelets are counted under the 1/6th in objective. The red cells should have been hæmolysed, leaving leucocytes and platelets stained blue, the latter appearing as pale blue refractile particles. The platelets are counted in an area of at least eighty small squares. If N be the number of platelets so counted, the number in one cubic millimetre will be $N \times 1000$

The above method is fairly satisfactory. It has the advantage that the platelets are counted directly. The platelets do not seem to agglutinate, but they are slow to settle in the counting chamber and may be difficult to see. The red cells sometimes hæmolyse slowly; in general it is best to leave the preparation in the damp chamber for an hour before counting the platelets. The method is useless in the presence of many reticulocytes, for basophilic material is liberated when the reticulocytes are hæmolysed

Other bodies which may be mistaken for platelets include fragments of leucocyte cytoplasm as sometimes seen in leukæmia, particularly in monocytic leukæmia, and "Pappenheimer bodies" (see p. 23). Cytoplasmic fragments cannot be distinguished with certainty from platelets, and in their presence this method of platelet enumeration breaks down entirely "Pappenheimer bodies" appear as smaller more intensely staining blue dots, and platelets can be separated from them without much difficulty.

It is essential that the pipette and counting chamber used should be absolutely clean and that the staining solution should be freshly filtered. The urea solution loses its hæmolytic power on keeping and should be renewed every week or so.

An "Indirect" Method for Counting Platelets in Venous Blood

In the method now described venous blood is added to a formol-citrate diluting fluid in a proportion of about 1·100, and the proportion of platelets to red cells estimated in a counting chamber.

Formula of Diluting Fluid. 1 per cent formalin in 3 per cent sodium citrate, as used for red cell counts. 1 ml. of a 0.5 per cent solution of brilliant cresyl blue in saline is added to each 19 ml of the above fluid, just before the count is undertaken. The mixture must be filtered before use

Method. Approximately 4–5 ml. of diluting fluid are filtered into a small waxed test tube provided with a rubber bung. A vein is cleanly punctured by means of a relatively wide bore needle and approximately 1 ml. of blood drawn into 1 ml of sterile 3 per cent sodium citrate solution contained in a small syringe lubricated with paraffin. The blood and citrate are mixed by inversion, and after detaching the needle, two or three drops of the mixture are dropped directly into the diluting fluid. The tube is corked and the suspension of blood mixed without delay by gently inverting the tube.

On return to the laboratory, the suspension is mixed by inversion for one to two minutes and a counting chamber filled using a waxed Pasteur pipette. After waiting about ten minutes for the platelets to settle, the total number of platelets in an area of 1 sq. mm., and the total of red cells in eighty small squares are counted. The 1/6th in. objective and ×6 or ×10 eyepiece should be used.

The platelets stain shades of blue and are usually well separated and can be readily seen.

If the total red cell count per c.mm. is known, the platelet count can be calculated from the ratio of platelets to red cells. Thus :—

Number of platelets in 1 sq.mm. area (400 small squares) =100
Number of red cells in 80 small squares =500

$$\text{Ratio of platelets to red cells} = \frac{100}{500 \times 5} = \frac{100}{2500} = \frac{1}{25}$$

If the red cell count was 5,000,000 per c.mm.,

$$\text{the platelet count would be } \frac{5{,}000{,}000}{25} = 200{,}000 \text{ per c mm}$$

The aim in the above method is to prevent agglutination of platelets as far as is possible. Contact with water-wettable surfaces is, therefore, reduced to a minimum Usually the platelets in the counting chamber are well separated, except in abnormally high counts when some clumps are almost always seen. Disintegration of the platelets is prevented by the fixing action of the formalin

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used for a red cell count and hæmoglobin estimation, etc

The Normal Range for Platelet Counts

The results obtained by the Lempert-Kristenson method and by the "indirect" method described above seem to be similar. The normal range in health by these methods is approximately 150,000 to 350,000 *per c.mm.*

It is a matter of personal preference which method is used. In either case experience and practice is required before much reliance

can be placed upon results. In the Lempert-Kristenson method,
although large numbers of platelets are av[illegible]
may be difficult to see [illegible]
In the indirect meth[illegible]
good preparation, rela[illegible] within a reasonable
time, and this is an additional *source of error* [illegible]
clinical purposes the general [illegible]
usually required. Certainly
differences in platelet cou[illegible]
degree of accuracy is unlik[illegible]
careful re[illegible]
worker
by Biggs

The "normal range" for platelet counts in the literature is astoundingly large, anything from 150,000 to 600,000 per c.mm. or more (2). Although undoubtedly there is a considerable variation from person to person, much of this wide range is due to differences in technique. The highest values are obtained by the use of the 1/12 in. objective, but there is no reason to suppose that high counts are necessarily correct ones; probably too many fragments of platelets or other particles are counted. Venous blood contains more platelets than does peripheral blood, and methods employing venous blood are to be preferred. Probably some platelets always adhere to the edges of a skin puncture wound, and are thereby lost.

Significance of the Platelet Count

All that can be said in a brief note is that a reduced platelet count is often, but not always, found in association with hæmorrhagic disorders characterized by spontaneous purpura and a prolonged bleeding time. The exact relationship between a deficiency in platelets and a tendency to bleed is, however, uncertain (5). A persistently reduced platelet count ($<$100,000 per c mm) is significant and may be a manifestation of a variety of pathological processes affecting the hæmopoietic tissues. A platelet count persistently raised above the wide normal range is less frequently found (6). The demonstration of either thrombocytopenia or thrombocythæmia is frequently of diagnostic importance.

THE COAGULATION TIME

The estimation of the time of the coagulation of blood *in vitro* is an artificial procedure, and the results depend very much upon the details of the method used. Amongst the variables which need to be controlled are the method of obtaining the blood, the size and nature of the vessel into which it is placed, the temperature of the blood, and how the blood is handled and the end point of coagulation read.

There is no doubt that the only reliable way to estimate the coagulation time is to use venous blood. A method based on that of Lee and White will therefore be described in detail The method of Dale and Laidlaw will be referred to because it is necessary to have available a method for peripheral blood, even if it is less satisfactory, should a clean vein puncture prove to be impracticable as it may be in small children.

Modification of Lee and White's Method for Venous Blood

Venous blood is withdrawn into a dry syringe and after detaching the needle, 1 ml. volumes are delivered into four clean rimmed tubes of 8 mm. internal bore previously warmed to 37°C. A stopwatch is started as soon as blood enters the syringe. The tubes are then gently tilted every minute in rotation until one can be tilted through an angle greater than 90° without the blood spilling. The remaining tubes are then examined at half minute intervals. The coagulation time is taken as the time necessary for the blood to clot in at least two of the four tubes.

Chemically clean tubes are essential. The vein must be cleanly punctured and a relatively wide bore needle used. The needle must fit well and no air bubbles should be withdrawn into the syringe. The needle should be detached before delivering the blood gently into the tubes , no froth must be delivered

It is important to control the temperature, as the speed of clotting increases with the temperature and is about twice as fast at 37°C. as at average room temperature (20°C.). For this purpose a wide-mouthed vacuum flask containing water

can be placed upon results. In the Lempert-Kristenson method,

degree of accuracy is unlikely to be achieved except by the most careful regard to detail, and then only in the hands of an experienced worker. The very large error in platelet counting is considered by Biggs and MacMillan (4)

The "normal range" for platelet counts in the literature is astoundingly large, anything from 150,000 to 600,000 per c mm. or more (2). Although undoubtedly there is a considerable variation from person to person, much of this wide range is due to differences in technique. The highest values are obtained by the use of the 1/12 in. objective, but there is no reason to suppose that high counts are necessarily correct ones; probably too many fragments of platelets or other particles are counted. Venous blood contains more platelets than does peripheral blood, and methods employing venous blood are to be preferred. Probably some platelets always adhere to the edges of a skin puncture wound, and are thereby lost.

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to and fro, and the movement of the shot noted. The coagulation time is the time which elapses between the moment of puncture and the arrest of the movement of the shot. It is convenient to view the shot against a light arranged behind the beaker.

A rapid flow of blood is absolutely essential, and if squeezing or manipulation is required to obtain the blood, the test should be abandoned. The method is undoubtedly insensitive and should not be used if venous blood can be obtained It may give apparently normal values at a time when the Lee and White method shows a definite prolongation in clotting time.

The *normal coagulation time* is up to three minutes.

Significance of the Measurement of Coagulation Time

The estimation of the coagulation time *in vitro* is only of limited value even if a careful technique is followed. The fact that only very small amounts of thrombin are required to clot fibrinogen means that the test is inevitably an insensitive one. For instance, the prothrombin concentration can be reduced to dangerous levels (about 20 per cent of the normal value) without necessarily raising the coagulation time outside the normal range, and fibrinogen has to be virtually absent before the blood fails to clot due to fibrinogenopenia.

In hæmophilia, however, the causal abnormality [probably a lack of plasma thromboplastinogen (8)] is usually such a severe one that the coagulation time *in vitro* is generally, although not invariably (7), prolonged: even at 37°C. coagulation may take two hours or more. The estimation of the coagulation time of a patient suspected of suffering from hæmophilia is thus an essential first step in the investigation of such a case

An *increased* coagulation time is not, however, necessarily indicative of hæmophilia. Rare instances of hæmorrhagic disorders clinically simulating hæmophilia more or less closely have been

at 37°C. is convenient, although a glass jar with a pierced metal lid can be used as a substitute. The tubes may be dropped through the holes in the cover or lid and supported by their rims. A separate hole for a thermometer is useful.

The practice of letting blood clot at room temperature is to be deprecated; coagulation is needlessly prolonged and the temperature itself is too variable. The size of the container must be standardized, for blood will clot faster in narrow tubes because of the increased surface of blood in contact with the wall of the container. The tubes must be perfectly clean and there is no point in trying to retard coagulation by using plastic (lusteroid) tubes or by coating them with silicone (7).

The exact measurement of the end point is a matter of some difficulty. Blood clots first at the surface exposed to air and at the periphery in contact with the glass container. It clots last in its centre. It is essential, therefore, in reading the end point to tilt the tubes gently and always in the same way if standard results are to be achieved. The complete coagulation of hæmophilic blood may take a very long time and the reading of the end point may be peculiarly difficult. Quick *et al.* (7) cite ten minutes as the time of commencing coagulation of a particular sample of hæmophilic blood, although coagulation was not complete enough to form a solid clot until two hours had elapsed.

Normal Range for Coagulation Times estimated by Lee and White's Method

Four to ten minutes (usually four to eight minutes) at 37°C.

Method of Dale and Laidlaw

A warmed ear or finger is deeply punctured, so as to obtain a free flow of blood. The coagulation tube, a piece of capillary 1.5 to 2 mm. in diameter and 15 mm. in length, with slightly constricted ends, and containing a small movable lead shot, is at once applied to the blood, which should fill the tube quickly and completely by capillarity. The tube is held at each end by rubber-covered forceps and is dipped into water at 37°C. contained in a glass beaker. The tube is gently tilted

known to have a prolonged bleeding time should be pricked with circumspection, and not too deeply, for once started the flow may be most difficult to stop. Within limits the size of the pricker is unimportant; it determines the volume of the blood rather than the duration of the flow, but if the puncture is too small the natural elasticity of the skin will seal the hole and prevent a proper flow.

Alternatively, the ear or pad of a finger may be pricked and the blood allowed to run into a small beaker containing saline warmed to 37°C. This has the theoretical advantage of keeping the part warmed. The patient can conveniently place the finger in a beaker. A continuous stream of blood should flow from the puncture. The end point is usually a sharp one, the cessation of flow being abrupt and usually following a short period during which the flow is reduced and jerky. The results by this method are very similar to the "dry" method outlined above.

Significance of the Bleeding Time

A skin puncture results in the severing of capillaries and sometimes probably small arterioles and venules. The length of time the puncture bleeds seems to be determined largely by the power of the vessels to contract and is more or less independent of the coagulation time of the blood. Whether or not the platelets play any role at all is uncertain (8).

A prolonged bleeding time is generally found in most of the bleeding disorders associated with spontaneous purpura; it is often but not invariably associated with thrombocytopenia. The test is unfortunately a comparatively crude one; although a skin puncture may bleed for hours and may even be a source of concern to the investigator and/or the patient—indicating without any shadow of doubt a hæmorrhagic disorder—the test is not a good one for demonstrating minor degrees of a bleeding tendency, for the range in health is so large.

An unexpected result should be confirmed or refuted by repeated observations before being necessarily accepted as correct.

from true hæmophiliacs, and the confirmation of hæmophilia itself require special experience. The study of the effects of the patient's plasma on the clotting of known hæmophilic plasma, the quantitative estimation of the consumption of prothrombin (12) and tests for circulating anticoagulants require to be carried out before a diagnosis can be made (13).

As has been mentioned, estimation of the coagulation time is usually of little value in the hæmorrhagic disorders due to hypoprothrombinæmia; it is, however, useful in controlling the therapeutic use of heparin (which probably acts as an antithrombin).

A *decreased* coagulation time is difficult to establish with certainty by ordinary methods, because of the difficulty in excluding technical causes. Stat[illegible]
however, do exist, for in[illegible]
likely that tests such as th[illegible]
ness of the anticoagulant [illegible]
more useful than the estimation of the crude whole blood coagulation time

BLEEDING TIME

The patient is seated on a chair or asked to sit upright in bed and his ear well rubbed with lint until quite warm. The lower part of the lobe is then sharply pricked with a mechanical pricker or a glass capillary pricker to a depth of 3 mm A stopwatch is then started and at half-minute intervals the drops of blood which exude from the cut are removed on to a piece of filter paper without touching the surface of the ear. Normally, the ear should cease bleeding within five minutes, although occasionally in health bleeding may not completely cease within ten minutes or even longer The cause of unusually long bleeding times in healthy people is obscure; possibly in some cases this is due to puncture of dilated venules, lacking the power to contract, which may be seen on the surface of some ear lobes

It is essential that the ear should be warm and that the blood flows spontaneously The maximum rate of flow is generally between ½ and two minutes after pricking; thereafter, the rate is abruptly slowed but may not cease for a further two or three minutes in a normal subject. A patient

carefully watched with the lower end just below the surface of the water. The development of a fibrin clot appearing quite suddenly within a second or so marks the end point. The test should be repeated once or twice more. The tube may then be lifted clear of the water a few seconds before the end point is expected in order to facilitate inspection. The average time for the appearance of the fibrin web is recorded as the prothrombin time. A normal control plasma should be tested at the same time in the same way.

In the above technique, dry oxalate mixture (16) is substituted for the sodium oxalate solution recommended by Quick, and human brain for rabbit brain. The advantage of using human brain is that large batches of thromboplastin can be made at the same time; if suitably stored, its potency will keep for months (see later)

The "prothrombin time" as estimated by the above method varies somewhat with the batch of thromboplastin, *but is usually from ten to fifteen seconds in normal subjects.* The observed prothrombin time can be converted into "prothrombin concentration" from conversion graphs made by artificially diluting plasma with saline or prothrombin-free plasma For a discussion of the rather doubtful justification for doing this, see Biggs and Macfarlane (17)

Despite being based on the fallacious assumption that the test is a measure of the amount of prothrombin present rather than [illegible] insensitive, it can be used despite uncertainty as to what is being measured to control the tendency to bleed of patients given dicoumarol for the treatment or prophylaxis of thrombosis It is essential, however, that the insensitivity of the test to reductions in prothrombin concentration should not be increased by the choice of thromboplastin used; if Russell's viper venom is used instead of brain extract, the prothrombin concentration may be reduced to dangerous levels before there is a significant prolongation of the prothrombin time (17, 18)

Estimation of Prothrombin; Preparation of Thromboplastin from Human Brain

A human brain freshly obtained from the postmortem room is stripped completely of its covering membranes and blood vessels.

ESTIMATION OF PROTHROMBIN BY A SINGLE-STAGE METHOD

[slightly modified from that of Quick (15)]

This test is widely used in clinical hæmatology, particularly in the control of dicoumarol therapy. The ~~principle~~ is that oxalated plasma is clotted by the addition of calcium in the presence of excess thromboplastin. The clotting time is then assumed to be directly related to the plasma prothrombin level.

Reagents Required. *Patient's and Control Plasma.* 5 ml. of venous blood is added to 10 mg. of dry potassium and ammonium oxalate mixture and gently mixed with the anticoagulant by several inversions of the tube. Part of the sample can be used for a red cell count, hæmoglobin estimation and hæmatocrit etc.; *the remainder should be centrifuged without* delay at 1500 rev. for five minutes. The supernatant plasma is removed at once and should be placed in the refrigerator at 2–5°C. until used. The test must be performed on the day of collection.

Thromboplastin Solution. It is best to use an acetone extract of human brain (for preparation see p. 121). 0 3 g. of the dry extract is suspended in 5 ml. saline and heated to 50°C. for ten to fifteen minutes before use. It is then centrifuged *lightly for one minute to deposit the coarse particles.* The opalescent supernatant is used for the test The solution may be kept for a day or two at 2–5°C. but no longer.

Calcium Solution M/40 aqueous calcium chloride is used (0.28 g. anhydrous calcium chloride dissolved in 100 ml. distilled water)

Technique

A 0.1 ml volume of the plasma to be tested is delivered to the bottom of a tube of 8 mm. internal bore and placed in a water bath at 37°C. and 0 1 ml. of thromboplastin solution added to it. After a delay of one minute 0.1 ml. of warmed calcium chloride solution is added and the contents of the tube quickly mixed. A stop watch is started and the tube

In anæmia, retraction is generally increased due to the smaller red cell volume, the relation between the two variables being approximately linear. The blood platelet level, below which impaired retraction may be expected, is said to be about 100,000 per c mm. Other conditions in which there may be impaired retraction include jaundice, pneumonia and myelomatosis.

Normal Limits of Clot Retraction [Macfarlane (19)]

In fifty subjects (27 men and 23 women) retraction varied from 48 to 64 per cent with a mean of 54.7. The statistical limits ($\pm 3\sigma$) were 44 to 65.5 per cent.

THE DIAGNOSIS OF THE CAUSE OF A HÆMORRHAGIC TENDENCY*

The following scheme indicates in summary form the order in which laboratory investigations should be undertaken:—

1. Hæmoglobin estimation and inspection of a stained blood film. The diagnosis may be obvious at this stage, as in leukæmia
2. A platelet count and total leucocyte count, and if there is anæmia, a red cell count and packed cell volume estimation.
3. Bleeding time, coagulation time and prothrombin time.

4a. If the bleeding tendency seems to be secondary to a blood disorder, as for instance in aplastic anæmia, any other investigations such as a reticulocyte count, sternal puncture, etc , which will help to elucidate the nature of the blood disorder, should be carried out

4b. If the bleeding tendency itself seems to be the primary disturbance, any tests which will throw light on the nature of the hæmorrhagic disorder should now be undertaken, e.g. prothrombin consumption test, tests for anticoagulants, microscopy of skin capillaries, etc

5. If indicated, tests to establish the presence or absence of avitaminosis C

It is then cut into pieces and macerated in acetone in a mortar. After the acetone has been changed several times a non-adhesive granular material remains. This is crude "thromboplastin."

The granular material is dried in an evacuated desiccator and when dry 0 3 g. amounts are placed in a number of 3 × ½in. tubes. These are stored in an evacuated desiccator at 2–5°C. until used. They retain their potency under these circumstances for months. The desiccator must be re-evacuated each time it is opened to remove a tube.

THE QUANTITATIVE MEASUREMENT OF CLOT RETRACTION

The relative failure of the clot to retract in blood from patients with a low platelet count has been known for fifty years or more. The degree of failure of retraction can be measured by the method of Macfarlane (19), which relates the volume of serum expressed to the original volume of blood.

Method of Estimation

Required A graduated centrifuge tube, reading to 0 1 ml, and *a glass rod about 5½ in. in length with a series of expansions* at about ½ in intervals, and with a perforated cork fixed at one end.

Technique. Rather more than 5 ml. of blood are withdrawn into a dry syringe by means of a clean venepuncture. It is at once delivered into a clean test tube without frothing and 5 ml. *delivered by pipette into the graduated centrifuge* tube. The glass rod is then placed in the tube and held in a vertical position by means of the cork

The centrifuge tube is then incubated at 37°C., preferably in a water bath, and examined from time to time until the blood has clotted firmly. It is then left undisturbed at 37°C for a further hour, at the end of which time the rod, around which the clot [illegible]

or a platinum loop and reincubated for a further hour, without introducing much error

In anæmia, retraction is generally increased due to the smaller red cell volume, the relation between the two variables being approximately linear. The blood platelet level, below which impaired retraction may be expected, is said to be about 100,000 per c mm. Other conditions in which there may be impaired retraction include jaundice, pneumonia and myelomatosis.

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5 If indicated, tests to establish the presence or absence of avitaminosis C.

* It is not contended for a moment that [illegible]

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CHAPTER 9

BLOOD GROUPS AND THE LABORATORY ASPECTS OF BLOOD TRANSFUSION

The human blood groups. Methods of ABO grouping Methods of Rh typing. The detection of Rh antibodies. Titration of agglutinins. The Coombs test. The direct matching test in blood transfusion The investigation of transfusion reactions.

No attempt will be made to give a detailed survey of the human blood groups nor to review the variety of techniques which may be used in the detection of antigens and antibodies. In this chapter will be described only some essential basic facts and selected practical techniques useful in the management of a hospital blood grouping service. For a more detailed exposition the reader is referred to the two British M R.C. Memoranda, "The determination of blood groups" (1) and "The Rh blood groups, and their clinical effects" (2) and to Wiener's monograph (3)

THE HUMAN BLOOD GROUPS

It is now known that human red cells contain many inheritable antigens or blood group factors, more no doubt still await discovery. Fortunately, however, only a few are of major clinical significance, the majority are of much less importance because normally they seem to have little antigenic power and antibodies are seldom developed against them. The important ones are the A and B factors, isoantibodies against which occur naturally, and the D (Rh) factor against which immune antibodies are quite frequently produced in people lacking this antigen, as the result of previous transfusion or pregnancy. All these antigens belong

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common Rh positive (D positive) genotypes (determined by a chromosome derived from each parent) are CDe cde $=R_1r$ (34.9 per cent), CDe CDe $=R_1R_1$ (18.5 per cent), CDe cDE $=R_1R_2$ (13.4 per cent), cDE cde $=R_2r$ (14.1 per cent) and cDe cde $=R_0r$ (2.1 per cent) (2). In addition there are about 15 per cent of persons of genotype cde cde $=$rr, referred to as Rh negative. The other possible genotypes occur, but less frequently; some are extremely rare.

Table IX

Possible combinations of Rh genes in chromosomes (in order of frequency)	Equivalent in Wiener's nomenclature
CDe	Rh_1 (R_1)
cde	r
cDE	Rh_2 (R_2)
cDe	Rh_0 (R_0)
cdE	Rh″ (R″)
Cde	Rh′ (R′)
CDE } very	Rh_z (R_z)
CdE } rare	Rh_y (R_y)

It should be emphasized that the great majority of cases of Rh incompatibility are due to the development of antibodies (anti-D) against the D antigen. The other Rh antigens are relatively weak ones, and although anti-C, anti-c and anti-E may occasionally be formed as the result of the transfusion of blood or of pregnancy, they are usually, but not invariably, associated with anti-D. Unlike the naturally occurring isoantibodies anti-A and anti-B, these Rh antibodies developing as the result of immunization often exist only in an "incomplete" form (see later)

The Less Important Blood Groups Antibodies against the other blood group systems mentioned above are rarely met with They have mostly been observed in patients who have received a series of blood transfusions, more rarely have they been developed

to groups or systems, of which the ABO, CDE cde, MNSs, Lewis, Lutheran and Kell systems are examples.*

The ABO Blood Groups. Although there are only four main blood groups involving A, B and O factors, i.e. groups AB, A, B and O, subgroups exist. Group A may be divided into A_1 and A_2 and AB into A_1B and A_2B; about 20 per cent of groups A or AB belong to the subgroups A_2 and A_2B respectively. The symbol O does not represent absence of A or B. A, B and O are probably co-dominant genes. Group A_2 is probably of genotype AA_1 and group A_2 of genotype AO (18).

Occasionally, agglutinins active against the subgroups of A are found; fortunately they are usually "cold" antibodies inactive at 37°C., and are thus of little importance. α_1 is met with in the sera of about 1 to 2 per cent of A_2 persons and in about 25 per cent of group A_2B. Anti-O, sometimes called α_2 because it reacts with 95 per cent of A_2 cells, as well as with O cells, is rarely found in the sera of A_1, A_1B or B subjects (see Table VIII)

Table VIII

Blood Group	Agglutinins normally present in serum	Agglutinins occasionally present
A_1	anti-B (β)	α_2 (very rare, reacts with A_2 and O cells)
A_1B	none	α_2 (very rare, reacts with A_2 and O cells)
B	anti-A (α)	α_2 (very rare; reacts with A_2 and O cells)
A_2	anti-B (β)	α_1 (1–2% of sera; reacts with A_1 or A_1B corpuscles)
A_2B	none	α_1 (25–30% of sera, reacts with A_1 or A_1B corpuscles)

The occurrence of the α_1 and α_2 agglutinins

The Rh Blood Groups. The Rh groups are classified in Fisher's nomenclature according to their content of three allelic antigens, CDE or cde (see Table IX). The most

* For details of the less common blood group systems the reader is referred to the forthcoming monograph of Dr R. R. Race and Miss R. Sanger.

should rarely occur if the sera are potent) may be checked by gently transferring the cell suspension to a slide and examining it under the 2/3rd in. or 1/6th in. objectives.

If a quick result is required and the tube method is used, the tubes may be centrifuged after standing for ten minutes at room temperature. On attempting to resuspend the deposit, clear-cut results are usually obtained. Agglutination causes the cells to stick together in a tight clump and only break up into coarse granules on vigorous tapping. Unagglutinated cells are easily resuspended into a smooth suspension. In case of doubt the suspensions may be examined microscopically.

A Rapid Slide or Tile Technique

Agglutination is much more rapid on flat surfaces. One drop each of a 5 per cent cell suspension, saline and grouping serum are mixed together on a slide or hollow tile and gently rocked. After five minutes or less the results may be read and are usually clear-cut and indisputable; they may be checked under the microscope. A minor objection to this technique is the slightly increased tendency to rouleaux formation. The method is nevertheless rapid and sensitive, and useful when a few samples are to be grouped.

The main ABO types give the following reactions —

Anti-A serum	+	+	–	–
Anti-B serum	+	–	+	–
	Group AB	Group A	Group B	Group O

Weak Reactions. The A agglutinogen in subgroups A_2 and A_2B is relatively insensitive to anti-A, and agglutination may be missed on this account unless high-titre sera are used. A common grouping error is to report group A_2B as group B.

Checking the Group by Testing for Agglutinins. Ideally in all cases, and whenever there seems any cause for doubt, the agglutinin content of the blood under examination should be tested for anti-A and anti-B by the methods outlined above. The donor's or patient's serum is substituted for the grouping serum and his cells replaced by known A and B cells.

in pregnancy and have been the cause of hæmolytic disease of the newborn. Like the Rh antibodies these rare antibodies often exist in an incomplete form (19). The importance of anti-M, anti-S and anti-Kell, etc., apart from their theoretical and genetical interest, lies in the fact that they are a cause, although a rare one, of unexplained hæmolytic reactions. Their possible presence is a reason why careful cross matching tests should always be carried out before transfusions, even if it is quite certain that both donor and recipient are of identical ABO and Rh groups.

METHODS OF A, B AND O GROUPING

The principle is that the corpuscles to be tested are exposed to the separate action of the anti-A and anti-B agglutinins obtained from group B and group A subjects respectively.

Sera used for grouping purposes must be of adequate titre, at least 128, and higher if possible. The blood should be allowed to clot under sterile conditions, and after the serum has separated, it should be centrifuged and the clear supernatant heated to 56°C. for half an hour, then Seitz-filtered and finally bottled in 2 ml. volumes. The sera must be kept frozen when not in use at as low a temperature as possible (preferably at -20°C. or below). It is essential that they are kept free from bacterial contamination, which may cause false positive agglutination (3, 5). Their potency should be checked from time to time with known A, B and O cells.

In grouping, two main procedures may be followed according to the urgency with which the result is required and the number of tests to be performed.

The Tube Technique

If there is no hurry and a large number of tests are to be performed, groupings may be carried out in tubes of 80×10 mm size or smaller. A 5 per cent suspension of the blood to be tested is made in 3 per cent sodium citrate One volume of this suspension (usually one drop) is placed in a pair of tubes to which are added one volume of saline and one volume of anti-A and anti-B sera respectively. The tubes are left for two hours at room temperature. The results are read visually after gently tapping the tubes. Doubtful reactions (which

theory it should be easy to distinguish rouleaux from true agglutination under the microscope; in practice this may prove difficult.

A serum containing *"cold" hæmagglutinins* should naturally not be used for diagnostic purposes, but difficulty, as with rouleaux formation, arises when normal corpuscles are suspended in a patient's serum in the course of a direct matching test. If "cold" agglutinins are known to be present, the test should be carried out at 37°C. and the patient's serum separated at 2–5°C. so that most of the agglutinin may be absorbed on to the patient's corpuscles at this low temperature.

An *infected serum* should never be used, not only are the specific agglutinins lost but it may acquire the property of agglutinating corpuscles of all groups. Control tests with known corpuscles provide an adequate safeguard, but this is an added reason why all sera should be replaced in the freezing compartment of the refrigerator as soon after use as possible.

Ideally, blood groupings should be performed with freshly collected corpuscles. If blood is stored, it should be kept in the refrigerator in the form of clotted whole blood. Should it become infected, the growth of certain bacteria may cause the corpuscles of blood of any group to become agglutinable by any human serum (5). This seems most likely to occur when diluted suspensions of blood are stored and is very rare when blood is kept in contact with its clot

METHODS OF Rh TYPING

For most practical purposes it is sufficient to classify the blood into Rh positive and Rh negative, using an anti-D serum 85 per cent of bloods are D positive.

A Method employing "Complete" Anti-D Serum

(Tube method)

As supplies of serum are usually limited, a micro-technique must be used, and because the titre may be unavoidably low (8 for instance) special care may be necessary in recording the results It is often convenient to use a low-titred serum

Determination of the Subgroups of A. As has already been mentioned, the subgroup A_2 may be suspected when a blood reacts weakly with anti-A of known potency. It may be more exactly determined in the following way:—

Anti-A sera from group O or B persons contain in effect two agglutinins, α and α_1. α reacts with A_1 and A_2 corpuscles and α_1 with A_1 corpuscles alone. If, therefore, a serum is absorbed with A_2 corpuscles, α is removed whilst α_1 remains. Such a serum will react positively with A_1 but not with A_2 corpuscles. The absorption is carried out by mixing equal volumes of washed A_2 corpuscles with the anti-A serum and centrifuging after allowing the mixture to stand for two hours at 2–5°C. When using the absorbed serum, known A_1 and A_2 corpuscles should be used as controls.

False Positive Reactions when Grouping for the A and B Factors

False positive reactions may result from several causes. Fortunately, the use of control corpuscles of known group will almost always distinguish false from true reactions. False positive reactions may be due to (1) the grouping serum having the property of causing rouleaux formation—such a serum should not be used for routine grouping, (2) the presence of "cold" panhæmagglutinins in the grouping serum or absorbed on to the corpuscles to be grouped ; in this case the patient may appear to be group AB, (3) the use of infected sera which may develop the property of agglutinating corpuscles of all groups and (4) the use of infected red cell suspensions.

Rouleaux formation is much more likely to cause trouble when normal corpuscles are suspended in a patient's serum during the course of a direct matching test prior to transfusion, rather than when patient's corpuscles are being grouped in normal grouping serum. Particularly is there this difficulty when as in multiple myelomatosis the level of serum globulin is high. In these cases the patient's own corpuscles should be used as an additional control. Rouleaux formation is usually more marked when the tests are carried out on tiles or slides rather than in tubes. Usually the rouleaux will disappear if the serum is diluted with one or two volumes of saline, but this is not always the case, depending as it does on the concentration of rouleaux-promoting proteins present. In

to give approximately a 2 cm. column. The tube is then dipped into the cell suspension and a column of similar length allowed to run in and make contact with the serum. After sealing the dry end of the capillary, it is set in plasticine so that the cell suspension is uppermost and tilted to an angle of 45°, or placed in a specially designed and inclined rack. The tube or rack of tubes is kept at 37°C. The cells will gradually sediment through the serum. If Rh negative, they will fall through evenly, and eventually form a solid column at the bottom; if Rh positive, they will form irregular clumps which will adhere to the tube giving an irregular beaded red layer along its lower side.

Results are usually definite within fifteen minutes. If there is any doubt, the tube may be left for a further fifteen minutes, and the sedimented cells then allowed to run on to a slide. After gentle spreading they may be viewed with a low power objective.

A possible cause of false positives is the use of insufficiently washed corpuscles in cases where marked rouleaux formation is taking place. If this is suspected, the corpuscles should be repeatedly washed in saline and the test set up again.

Other Methods of Rh Grouping: the use of "Incomplete" (in-)Albumin-Agglutinating Anti-D Sera

The comparative rarity of (in-)saline-agglutinating antibodies makes it sometimes necessary to use the "incomplete" form of antibody. In this case the suspensions of corpuscles are centrifuged and as much saline as possible removed by pipette. The corpuscles are then resuspended in a volume of 20 per cent bovine albumin* (8) and the grouping serum added. The tube is left for two hours at 37°C. and the cell suspension examined microscopically at the end of the time. Alternatively, the tube may be gently centrifuged after fifteen to thirty minutes to accelerate agglutination. The deposit is examined microscopically as before. Other possible methods and slide tests are outlined in the M.R.C. Memorandum (2).

Determination of the Rh Genotype. It is of some practical importance to know the genotype of the husband of a woman

* Manufactured by Armour Laboratories

as a screening test and to re-type any doubtful and negative reactions using a better serum kept for this purpose.

The sera, usually obtained from a lately pregnant woman a week or so after delivery of a child with hæmolytic disease of the newborn, should be absorbed with A or B Rh negative cells, as may be necessary, so that they may be used with all blood groups. Equal volumes of serum and washed packed A and/or B cells are mixed together in equal proportions and allowed to stand in the refrigerator at 2–5°C. for two hours. After centrifugation the absence of anti-A and anti-B must be confirmed by testing with A or B Rh negative cells. If traces of anti-A or anti-B are still present, the process must be repeated. The sera should finally be Seitz-filtered and stored in sterile containers in 1 ml. or 2 ml. volumes. They should be kept frozen at as low a temperature as possible.

In the test, equal volumes (one small drop) of a 2 per cent suspension of cells and serum are each delivered to the bottom of a 50 × 6 mm. tube. The cell suspension is mixed with the serum and the whole incubated at 37°C. for two hours. The results may be read first by viewing the bottom of sedimented cells in a concave mirror—a negative result will be shown by a perfectly smooth-edged small round button, a positive by a larger deposit of cells with irregular or fluffy edges. Doubtful reactions and apparent negatives should then be examined under the low powers of the microscope after carefully transferring the deposit to a slide and gently spreading it out with a sealed glass capillary. As the clumps of agglutinated cells are easily broken up, at least using sera of low titre, care and experience may be necessary in the interpretation of results. A temperature of 37°C. is desirable as agglutination is accelerated.

An Alternative Method employing "Complete" Anti-D Serum (Chown's capillary tube method)

The advantages of this most useful method are economy of serum and the rapidity with which results may be obtained. A 15–20 per cent suspension of corpuscles in saline is required.

Small glass capillaries of about 0.5–1.0 mm. bore are used, and enough grouping serum allowed to run in by capillarity

TITRATION OF THE AGGLUTININ CONTENT OF SERA

Anti-A and Anti-B Agglutinins. The method for testing for the anti-A and anti-B agglutinins using known A and B corpuscles has already been described. The titre of the agglutinins may be estimated by making serial halving dilutions of the serum in saline and adding to each dilution an equal volume of a 1 per cent suspension of washed A* or B corpuscles. 0 15–0 25 ml. volumes are convenient. The number of tubes required depends on the potency of the serum; ten tubes are usually sufficient as a titre greater than 512 (1024 final dilution) is seldom found. A saline control should always be set up The corpuscles are mixed with the serum dilutions and the whole series left undisturbed for two hours at room temperature. End points may be read by a combined macroscopic and microscopic method, or simply macroscopically, after a standard degree of tapping of the deposit or inversion of the tubes. The degree of agglutination ranging from complete (C) to weak (±) should be scored as described in the M R C War Memorandum No. 9, "The Determination of Blood Groups" (1) The end point (±) is where there is a uniform distribution of small clumps of up to four to six corpuscles in a field of unagglutinated red cells.

Rh Agglutinins. "Complete" or (in-)saline-agglutinating antibodies may be titrated in a similar way to anti-A and anti-B In this case, however, small 50 × 6 mm. tubes should be used in order to economize the serum, and Rh positive corpuscles containing the appropriate antigens employed, e g R_1R_1 or R_1R_2 (CDe CDe or CDe cDE). The series of tubes should be capped and incubated at 37°C. for two hours The end point of agglutination may be read by inspection of the pattern of deposited cells as seen in a concave mirror and confirmed by gentle transference of the deposits to slides for examination under the microscope

The "incomplete" or (in-)albumin-agglutinating form of Rh antibody cannot be titrated in saline, for though it is

* Because of their greater agglutinability corpuscles of subgroup A_1 should be used rather than those of subgroup A_2

who has developed Rh antibodies. *If he is, for instance, homozygous* in respect of the D gene and the antibody present in the mother's serum is anti-D, then all his children will be affected. If, however, he has but one D gene and is thus heterozygous, only half his children will be affected.

The determination of the genotype is a task for a specialist laboratory, for the sera required are rare and precious. The principle [illegible] Rh [illegible] that *minimal volumes of sera are employed.* The results are read microscopically. In the absence of anti-e (rare) and anti-d (almost unknown), it is only the probable genotype that can usually be determined, the choice often being between a common type and one or more very rare combinations. Sometimes knowledge of *the reactions of other members of the family will help (2).*

Detection of Rh Antibodies

The methods for the detection of Rh antibodies are the same as those used in Rh grouping, with the difference that the unknown serum is substituted for the known grouping serum and Rh positive corpuscles substituted for the unknown corpuscles. In general it is best to use R_1R_2(CDe cDE) corpuscles of good agglutinability. For the detection of rarer antibodies such as anti-C, or anti-E, corpuscles of R' or R" (Cde or cdE) are useful, but are seldom available. As the incomplete antibody is more frequently found, it is essential if saline agglutinins are absent, to resuspend the corpuscles in 20 per cent albumin after being in contact with the serum under test. As a screening test it is often convenient as a first step to add one volume of serum to one volume of R_1R_2 corpuscles suspended in albumin. If the reaction is positive, *the serum can be tested later for saline-agglutinating antibody.* *

Occasionally, a zone phenomenom results in inhibition or but a trace of agglutination using undiluted serum and a saline suspension of corpuscles. Inhibition of agglutination in albumin is much rarer, but is not unknown. In these cases the presence of agglutinins *will only reveal itself if the serum is diluted.* ~

* Morton and Pickles (9) have demonstrated that normal Rh positive

from its power to interfere with the agglutinative properties of the "complete" normal type of antibody may be tested for

rabbits which have been immunized against human globulin [Coombs, Mourant and Race (11)].

The Coombs test* is used in detecting the presence of "incomplete" antibodies in serum and in the diagnosis of hæmolytic disease of the newborn by demonstrating that the infant's corpuscles have absorbed the antibody. Its use in the detection of antibody absorbed by the corpuscles of patients suffering from idiopathic acquired hæmolytic anæmia in adults has already been referred to.

The test is performed by well washing the corpuscles under test in several changes of a large volume of saline. Two separate drops of an approximately 5 per cent suspension of the washed corpuscles are then transferred to a glass slide or opalescent tile. A drop of anti-human-globulin serum and a drop of saline (as control) are added to and mixed with the two drops of red cell suspension respectively. The tile may be held over an electric lamp to warm it, and should be gently rocked from time to time (13). If the test is positive, well marked agglutination will be observed within six minutes, often within one minute A positive control using corpuscles known to have absorbed "incomplete" antibody and a negative control using normal corpuscles should be put up at the same time

The test as described above is the "direct test" When used to demonstrate the presence of incomplete antibody in serum, it is known as the "indirect" test. Rh positive (D positive) corpuscles are suspended in serum or in dilutions of it in saline, as in titrating saline-agglutinating antibody After the suspension has stood for one to two hours at 37°C., the corpuscles are well washed in saline and the rabbit serum added as described above

The Coombs test is an extremely sensitive method of detecting sensitization, *provided that a potent rabbit* serum is available. A positive Coombs test may, in fact,

* This test has been referred to in America as the "Developing test" (12)

readily absorbed when diluted with saline, it does not then cause agglutination. A high concentration of protein is necessary. It may be titrated in the usual way using 20 per cent bovine albumin solution in place of saline as the medium for diluting the serum and suspending the red cells, or by titrating in saline in the usual way and centrifuging after allowing the suspension to stand for one to two hours at 37°C. so that the corpuscles may absorb any antibody present. The supernatant saline is removed by pipette as completely as possible and replaced with a volume of 20 per cent albumin. The corpuscles are then resuspended in the albumin and the series of tubes is reincubated for two hours. The titre of agglutination is read microscopically after transferring the deposited corpuscles on to slides. Alternatively, the suspensions in albumin may be centrifuged after half an hour's incubation and agglutination looked for microscopically after gently resuspending the deposits. Human group AB plasma or serum may be used in place of bovine albumin, or with the addition of a small proportion of albumin (10). Plasma is a better medium than serum in revealing agglutination, but both are probably less satisfactory than is albumin.

A further alternative is to perform a Coombs test on the corpuscles after they have been suspended and sensitized in serum diluted with saline. After the titre (if any) in saline has been read, the tubes are centrifuged, and the deposits washed three times in a large volume of saline and then tested for adsorption of antibody by the use of anti-human-globulin rabbit serum (see below).

THE COOMBS TEST

The "incomplete" or (in-)albumin agglutinating form of Rh antibody,* sometimes called the blocking antibody

* It is probable that the incomplete Rh antibody (and possibly other

observed

addition it should be able to be performed quickly if speed is necessary, and therefore must not be too elaborate.

A direct matching test has two functions; to guard against gross mistakes in grouping—almost any test will do this, and to pick up even traces of unusual and unexpected antibodies, a much more exacting task. It is only in women who have borne children and in men and women who have been previously transfused that these latter antibodies are at all likely to be found. In these people especial care in matching must be taken. The albumin method may occasionally fail to detect some types of "incomplete" antibodies (see footnote to p. 136) It is probably best, therefore, to carry out an indirect Coombs test in such cases, if a potent anti-globulin serum is available.

Method. The following method is recommended for routine use Approximately 0.2 ml. of a 3 per cent suspension of the donor's blood in saline is delivered into a small serological tube and centrifuged. As much as possible of the saline is then removed by pipette leaving an almost dry button of cells. To this button is then added one volume (0.1 ml. approximately) of the patient's serum and one volume of 20 per cent albumin. The preparation may be capped and left at 37°C. for two hours or centrifuged after fifteen to thirty minutes. After gentle suspension of the deposit, part of the corpuscle suspension is transferred to a slide and viewed under the microscope If there is definite agglutination, the blood must not be used. If any aggregation present is thought to be due to rouleaux formation 2–3 volumes of saline should be added, and the cell suspension again viewed under the microscope—rouleaux formation should be abolished by this procedure. If there is still doubt, probably the best thing to do is to wash the sensitized corpuscles well with saline and to perform a Coombs test. A positive reaction will be proof that the clumping was due to true agglutination.

When there is no question of the presence of "incomplete" antibodies, e.g in first transfusions or in women who have not borne children, a very simple tube test using a weak suspension of corpuscles in saline and a 1 3 dilution of serum may be employed.

1 volume of saline and 1 volume of a 5 per cent red cell suspension

be the only sign of hæmolytic disease of the newborn and a positive reaction may persist for many weeks after birth* (13). ✓

[illegible] Rabbit Serum

[illegible] serum is best made by a slight [illegible] Proom (14). Alum-precipitated serum is used. 25 ml. of human group O serum are diluted with 50 ml. distilled water and 90 ml. of 10 per cent aqueous potash alum added. The *p*H is adjusted to 6.5 and the precipitated proteins washed twice with saline The final precipitate is suspended in 100 ml. saline containing 1/10,000 *methiolate* as preservative.

5 ml. of this antigen are injected intramuscularly into each hind leg of a rabbit. The injections are repeated after 14 days *and the rabbit bled* (by heart puncture) after a further 10 days The rabbit's serum is inactivated at 56°C. for 30 minutes and then absorbed with a [illegible] A_1B and O red cells. [illegible] tested by means of [illegible] serum causes any agglutination, the absorption must be repeated, if necessary more than once.

Rabbits vary *considerably in their power of making a good* antiglobulin serum; in some animals more than two injections of protein are required Alternatively, sera may be made by giving a course of intravenous and intraperitoneal injections of whole serum (2)

THE DIRECT MATCHING TEST IN BLOOD TRANSFUSION

It is essential that a *direct matching* test is undertaken between the donor's red cells and the recipient's serum in any case of blood transfusion It is essential even if there is no question as to the blood groups of recipient and donor The test should ascertain compatibility in respect of the A, B and O system, and should be capable of revealing the presence of even small concentrations of both "complete" or "incomplete" antibodies It should also enable the observer to distinguish between serious incompatibility on the one hand and "cold" agglutination and rouleaux formation on the other. In

* A positive Coombs test may be observed at a time when there is no evidence that hæmolysis is proceeding at an increased rate. The significance of this observation is not clearly understood

addition it should be able to be performed quickly if speed is necessary, and therefore must not be too elaborate.

A direct matching test has two functions; to guard against gross mistakes in grouping—almost any test will do this, and to pick up even traces of unusual and unexpected antibodies, a much more exacting task. It is only in women who have borne children and in men and women who have been previously transfused that these latter antibodies are at all likely to be found. In these people especial care in matching must be taken. The albumin method may occasionally fail to detect some types of "incomplete" antibodies (see footnote to p. 136). It is probably best, therefore, to carry out an indirect Coombs test in such cases, if a potent anti-globulin serum is available.

Method. The following method is recommended for routine use. Approximately 0 2 ml of a 3 per cent suspension of the donor's blood in saline is delivered into a small serological tube and centrifuged. As much as possible of the saline is then removed by pipette leaving an almost dry button of cells. To this button is then added one volume (0 1 ml approximately) of the patient's serum and one volume of 20 per cent albumin The preparation may be capped and left at 37°C for two hours or centrifuged after fifteen to thirty minutes. After gentle suspension of the deposit, part of the corpuscle suspension is transferred to a slide and viewed under the microscope. If there is definite agglutination, the blood must not be used If any aggregation present is thought to be due to rouleaux formation, 2–3 volumes of saline should be added, and the cell suspension again viewed under the microscope—rouleaux formation should be abolished by this procedure If there is still doubt, probably the best thing to do is to wash the sensitized corpuscles well with saline and to perform a Coombs test. A positive reaction will be proof that the clumping was due to true agglutination

When there is no question of the presence of "incomplete" antibodies, e g in first transfusions or in women who have not borne children, a very simple tube test using a weak suspension of corpuscles in saline and a 1 3 dilution of serum may be employed

1 volume of saline and 1 volume of a 5 per cent red cell suspension

are added to 1 volume of the patient's serum. The mixture may be left for 15 minutes or longer at 37°C., then centrifuged and the effect of gentle tapping observed. Centrifugation makes the test very sensitive; "complete" or normal agglutinins of weak potency will be revealed, but "incomplete" agglutinins will be missed. Rouleaux formation will be inhibited by the saline.

The practice of incubating the suspension at 37°C. has been recommended, so as to avoid the complication of agglutination due to "cold" antibodies. If, however, they are present at high titre and have a high thermal amplitude, *their presence should be made known to the clinician so that* the blood transfused may be warmed and the risk (? only theoretical) of the recipient agglutinating cold corpuscles as he receives them, removed

In general, it is best to employ a single test only, as far as possible, suitable for all circumstances. If this policy is adhered to, the first test mentioned, employing albumin, should be used and the alternative abandoned. In any case considerable experience and care are required; for although nine out of ten reactions, at least, will be straightforward, every now and again slight agglutination maybe encountered, the significance of which may prove most difficult to determine

THE INVESTIGATION OF TRANSFUSION REACTIONS

Transfusion reactions vary widely in type and in severity. They have many different causes, and *considerable experience* and sometimes special techniques may be required to elucidate them. Broadly speaking they can be classified into two main groups, those produced by serological incompatibility resulting in hæmolysis (15), and those not due to this cause. In the second category are included a wide variety of conditions; they include febrile reactions due to pyrogens in the anticoagulant solution or apparatus, urticarial or other reactions due to sensitivity to protein, cardiac embarrassment due to too rapid transfusion, and sudden death due to air embolism. A consideration of cases of this sort is beyond the scope of this book The laboratory worker is deeply con-

cerned, however, in the investigation of reactions in order to establish firstly, whether any particular reaction is due to hæmolysis and secondly, if hæmolysis has taken place, to find out why this has occurred.

The incident should be investigated more or less as follows, depending upon the circumstances :—

1. The transfusion should be stopped, or at least brought almost to a standstill, as soon as there is a suspicion that there may be something seriously wrong, and that this may be due to some kind of incompatibility leading to hæmolysis.

2. A sample of venous blood should next be collected from a vein well away from the transfusion site. This blood should be withdrawn slowly into a dry syringe and delivered into a bottle containing heparin or a small volume of 3 per cent sodium citrate solution. Every care should be taken to avoid hæmolysis during collection. Instructions should be given that the next specimen of urine passed by the patient should be saved and the blood bottle preserved if not yet disconnected from the patient. Enquiry should be made as to how the blood has been treated ; whether it has been warmed and how long it has been out of the refrigerator, etc

3. Part of the sample of blood withdrawn from the patient should at once be centrifuged and the supernatant plasma inspected If there is no evidence of free hæmoglobin or obvious increase in bilirubin, it is not likely that there has been any serious degree of hæmolysis (but see later, 4c). The presence or absence of oxyhæmoglobin or increased bilirubin in the plasma depends naturally on the rate the blood is being transfused, the volume transfused before the sample is taken and the rate at which hæmolysis is taking place. A sample of the donor's blood should also be centrifuged and the supernatant plasma inspected for hæmolysis ; if there is any suggestion that the blood has been infected, it should be cultured. The age of the blood should be ascertained

4. If it seems likely that hæmolysis due to serological incompatibility has occurred, the next step is to try to establish the mechanism This should be done in several stages.

(a). The donor and recipient's blood should be re-grouped

and the crossmatching test performed again. These tests may show straightaway that some gross error has been committed. For the purpose of the crossmatching test, pre-transfusion serum must be used; if serum withdrawn after transfusion is employed instead, incompatibility may be missed because the causal agglutinins may have been completely absorbed *in vivo* by the donor's blood. Similarly, pre-transfusion corpuscles should be used for re-grouping the patient if they are available. Confusion may arise if blood is used which has been withdrawn from the patient after a reaction due to incompatibility has occurred. Under these circumstances it is possible for a group A subject, transfused with B cells, to appear to belong to group AB and an Rh negative person transfused with Rh positive cells to appear to be Rh positive, because both the recipient and donor's corpuscles may be present together. If it seems possible that hæmolysis may have been due to the presence of an antibody of the "incomplete" type, the matching test should be carried out in the presence of albumin or a Coombs test performed on the corpuscles after suspension in the patient's serum.

(*b*) If the above tests are clearly negative, it is unlikely although not impossible, that the hæmolytic reaction is due to hæmolysis of the donor's corpuscles. The next step is to consider the possibility that hæmolysis of the patient's corpuscles has taken place, perhaps due to anti-A or anti-B at high titres being transfused in O blood given to A or B recipients. The anti-A or anti-B titre of the transfused plasma should, therefore, be ascertained; a titre >500 will often cause minor degrees of hæmolysis (16), depending upon the rate transfused and the amount Higher titres may cause serious reactions *

(*c*). It may be possible to confirm the nature of the hæmolytic reaction by estimating the survival of the transfused corpuscles by means of the differential agglutination method (15) By the use of anti-A, anti-B, anti-M or anti-Rh it is often possible to demonstrate unequivocally that the

* In nocturnal hæmoglobinuria the patient's red cells are extraordinarily sensitive to isohæmolysin and perhaps other factors in plasma Even small volumes of plasma may precipitate a hæmolytic episode (17)

transfused corpuscles are present in the expected numbers (approximately 300,000–400,000 per c.mm. in adults per 500 ml. citrated blood), or that far fewer than expected are present, or that all have disappeared. By counting the total number of red cells as well as the number of the transfused red cells, it may even be possible by employing this technique to prove without doubt that the hæmolysis has been due entirely to destruction of the patient's own corpuscles (17) Use of these techniques will also demonstrate minor or "silent" degrees of hæmolysis of insufficient rapidity to cause oxyhæmoglobinæmia or a significant rise in plasma bilirubin.

(*d*). If the patient is first seen after the incident and no donor blood is available, the nature of the hæmolytic reaction may be revealed by an increase in titre of an isoagglutinin in the patient's serum; e.g. a many-fold rise in anti-A in a group B subject reaching a peak one to two weeks after transfusion is a clear indication that the recipient had received group A blood (15)

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Chapter 10

MISCELLANEOUS INVESTIGATIONS

Estimation of the erythrocyte sedimentation rate (E.S.R.) The Paul-Bunnell heterophile agglutination test. Preparation and staining of "thick" blood films.

ESTIMATION OF THE ERYTHROCYTE SEDIMENTATION RATE (E.S.R.)

Although an empirical test, the estimation of the erythrocyte sedimentation rate has been widely used in clinical medicine. Many methods for its measurement have been devised (1, 6), differing in respect of the anticoagulant used, the volume of blood employed, the dimensions of the tube in which the measurement is carried out, the time allowed for sedimentation to take place and the method of recording the results. The method described in the following section is that of Wintrobe and Landsberg (2). It is convenient to use because a measurement can be carried out as a preliminary to estimating the packed cell volume in the course of a hæmatological investigation. The method has the disadvantage, shared by many other methods, of employing a short (100 mm.) column of blood. The maximum sedimentation of non-anæmic blood is thus in the region of 50 mm.

Method. 5 ml. of venous blood are delivered into a tube containing 10 mg. of dry potassium and ammonium oxalate mixture. The sample is well mixed and a Wintrobe hæmatocrit tube filled to the 100 mm. mark. This is placed exactly vertical and left undisturbed for sixty minutes, after which time the level of the top of the column of sedimented red cells is measured. This figure subtracted from 100 mm. represents the length of the column of supernatant plasma and is the "sedimentation rate," usually recorded as so many mm. per hour.

The hæmatocrit tube may then be centrifuged for half an hour at 3000 rev. per minute and the packed cell volume ascertained. The sedimentation rate should be "corrected" for anæmia using Wintrobe and Landsberg's chart (2) and values obtained corresponding with packed cell volumes of 47 and 42 *per cent in men and women respectively.*

Normal Range (2)

Men. Mean = 3 7 mm. : range $\pm 2\sigma$(95% of observations) 0–9 mm.

Women. Mean = 9 6 mm. : range $\pm 2\sigma$(95% of observations) 0–20 mm.

The phenomenon of erythrocyte sedimentation has been exhaustively *investigated ; ultimately, it is a problem of physical* chemistry (1, 3)

The rate of fall of the red cells depends very largely upon their power of forming rouleaux, large clumps which sediment much more rapidly than single cells. This property is mainly controlled by the concentrations of fibrinogen and to a lesser extent of globulin in the plasma. Defibrinated blood sediments extremely slowly, not more than a mm. an hour, unless there is a pathologically raised globulin level, or an unusually high globulin-albumin ratio Red cell factors, however, also play a part (4)

Sedimentation can be observed to take place in three stages; a preliminary stage of at least a few minutes during which time the rouleaux form, followed by a period in which the sinking of the rouleaux takes place at approximately a constant speed and finally a phase during which the rate of sedimentation slows as the rouleaux pack towards the bottom of the tube. It is obvious that *the longer the tube that is used, the longer* will be the second period and the greater will sedimentation appear to be With rapidly sinking rouleaux a reading may have to be taken long before an hour has elapsed, and in these cases serial readings are useful Probably the most satisfactory method of reading is one in which multiple readings are taken during the second period and the maximum rate of fall calculated (6, 8)

Anæmia by altering the ratio of red cells to plasma encourages rouleaux formation and accelerates sedimentation Wintrobe's method of correction for anæmia has been criticized—it is possible to obtain negative values for the "corrected sedimentation rate," but the *principle* is on the whole useful [see also Hynes and Whitby (5)]. Dilution with sodium citrate, as is practised in Westergren's method, results in a slightly slower rate of fall.

Variations in Results Due to Technical Factors. Anticoagulants such as heparin (up to 0.2 mg per ml), ammonium and potassium oxalate mixture (1 to 3 mg per ml) and potassium oxalate (2 mg per ml.) do not affect the rate of sedimentation when compared with hæmophilic blood to which no anticoagulant is added A rise in temperature results in increased sedimentation as does even a small deviation of the tube from the vertical. The diameter of the tube should be 3 mm or greater, and the height not less than 100 mm. These and other modifying factors are considered in full by Ham and Curtis (1) and reviewed by Nichols (6) A poor delineation of the upper layer of red cells, so called "stratified sedimentation," has been attributed by Stephens (7) to the presence of many immature cells

THE PAUL-BUNNELL TEST FOR HETEROPHILE ANTIBODY

The presence of anti-sheep cell hæmagglutinins at unusually high titres in the sera of patients suffering from glandular fever (infectious mononucleosis) was described by Paul and Bunnell in 1932. Absorption methods for differentiating between the glandular fever antibodies and the natural ones, and those of serum sickness, have since been reported. The technique described below is based on that of Barrett (9)

The following reagents are required :—

1. 1 ml. of patient's serum inactivated by heating to 56°C. for half an hour.
2. Guinea pig-kidney emulsion, for preparation, see later.
3. A 2 per cent ox-cell suspension, for preparation, see later
4. A freshly prepared 0.4 per cent suspension of washed sheep cells in saline. The sheep blood should not be more than seven days old, and should have been stored at 2° to 5°C

Method

Three × 0.25 ml. volumes of inactivated patient's serum are delivered into three small tubes, A, B and C. 1 0 ml. saline is added to tube A, 0 75 ml. saline and 0 3 ml guinea pig-kidney emulsion to tube B, and 1 ml. of ox-cell suspension to tube C. The contents of the three tubes are mixed and then

placed in the refrigerator at 2 to 5°C. for at least two hours, or overnight. The tubes are then centrifuged and the supernatants retained. One in five dilutions in saline of unabsorbed serum and serum absorbed with guinea pig kidney and ox cells respectively are thus available.

A Short Screening Test. 0.25 ml. of 0.4 per cent sheep-cell suspension is added to 0 25 ml. of the guinea pig-kidney absorbed serum (from tube B). After leaving the tube at room temperature for fifteen minutes, it is centrifuged for two minutes at 1000 rev. per minute. An attempt is then made to resuspend the deposit by gently tapping the tube. If the deposit resuspends easily and there is no macroscopic agglutination, the whole test is considered to be negative. If there is agglutination, further steps should be taken.

The Quantitative Test. Serial halving dilutions of the sera from tubes A, B and C are made in saline. Ten tubes and a control containing saline should be put up as a general rule. An automatic pipette delivering approximately 0.2 ml. volumes is invaluable. Equal volumes of the 0 4 per cent sheep-cell suspension are then added to each tube, giving final serum dilutions of from 1:10 (tube 1) to 1:5120 (tube 10).

The tubes are incubated for one hour at 37°C. and then left undisturbed at room temperature for an additional hour or more so that the cell suspensions may sediment completely Alternatively, they may be left overnight in the refrigerator at a temperature above freezing point. If they have been in the refrigerator, they should first be allowed to regain room temperature before the endpoint is ascertained.

If the agglutinated cells have been allowed to sediment undisturbed, the presence of agglutination is most accurately revealed by the pattern of the deposit, a perfectly smooth button being seen in the saline control and varying degrees of dispersion and irregularity in the positive tubes *After a preliminary inspection* of the deposits has been made, the end points of each series should be read as the last tubes showing detectable agglutination compared with the controls after the deposit has been resuspended by gently inverting the tubes 2 3 times The resuspended cells may be viewed in a concave mirror or the presence or absence of agglutination determined under the microscope.

The following figures are given as examples of typical results :—

Unabsorbed serum, end point tube 7 ; titre 640.
Guinea pig-kidney absorbed serum, end point tube 7 ; titre 640.
Ox-cell absorbed serum, end point tube 4 ; titre 80.

Such a result would be *positive* for glandular fever ; the antibody being *not* absorbed by guinea pig kidney and (partially) absorbed by ox cells. The naturally occurring antibody is absorbed by guinea pig kidney and not by ox cells, and that of serum sickness is absorbed by both reagents.

Unabsorbed serum, end point tube 3 , titre 40.
Guinea pig-kidney absorbed serum, no agglutination in tube 1.
Ox-cell absorbed serum, end point tube 3 , titre 40

This is a normal result, and in the above case the screen test would have been negative The absorbing power of the guinea pig-kidney emulsion and the ox cells should be tested with positive and negative sera from time to time Barrett (9) states that each will keep for at least six months if stored in a refrigerator.

The antibodies normally present in human sera against sheep red cells are of the Forssman type, i e antibodies reacting against an antigen widely spread in animal tissues Antibodies of this type are formed by rabbits injected with an emulsion of guinea pig kidney The antibodies will be found to react with horse, dog, cat and mouse tissues as well as with sheep corpuscles, but not with the tissue of man, ox or rat, etc (11) The antibody formed in glandular fever is of a different nature and is not

Interpretation of Results

The Paul-Bunnell test is generally looked upon as a most

useful aid to the diagnosis of glandular fever, but like most tests it is not one hundred per cent reliable. Antibodies are often present as early as the fourth to sixth day of the disease (12) and are almost always found by the 21st day (13). They disappear as a rule within four to five months (12). Most authors have reported 80–90 per cent of positive results in cases of glandular fever (13).

Two points need further consideration; (1) the frequency of negative reactions in glandular fever, and (2) the limits of the normal and the specificity of positive reactions.

There is, unfortunately, no unanimity as to how frequently negative reactions are found in true glandular fever, or indeed as to whether negative reactions are ever found It has been shown that occasionally the characteristic antibodies develop very late in the course of the disease, perhaps weeks or even months later (13); and it is also known that a positive reaction may be transient and that the antibodies may be present at such low titres that they may be missed or may produce anomalous reactions when associated with the naturally occurring antibody at similar titres For all the above reasons it is difficult to state categorically that any particular patient has not or will not produce antibodies

The limits of the titre of heterophile antibodies present in health are ill-defined, largely because of differences in the technique used to demonstrate them Fortunately this is of little consequence, because in the diagnosis of glandular fever it is only the antibody not absorbed by guinea pig kidney which is of significance

Using the technique described above, the screening test
ilution of 1 10 or more after
was positive 39 times out of
Postgraduate Medical School
of London Twenty-two of the positive tests were given by sera from patients diagnosed with confidence on clinical and hæmatological grounds as suffering from glandular fever; of the remaining 17 reactions, one was from a patient suffering from monocytic leukæmia three others were from patients in which the diagnosis of glandular fever seemed improbable but not impossible, the remaining positive reactions were from patients in whom the diagnosis was probably but not certainly glandular fever The titre in the one false positive reaction was 10, and in the three other possibly false positive reactions also 10

The following are examples of anomalous reactions considered, however, to be positive, and apparently due to the glandular fever antibody and the normal antibody being both present at low titres :—

		a. 16.3.49	b. 21.3.49
Unabsorbed serum . . .	titre	40	80
Guinea pig-kidney absorbed serum . . .	titre	10	10
Ox-cell absorbed serum .	titre	20	80

This case was clinically and hæmatologically typical of glandular fever.

		11.4.49	14.4.49	22.4.49
Unabsorbed serum .	titre	160	80	160
Guinea pig-kidney absorbed serum .	titre	20	10	20
Ox-cell absorbed serum	titre	40	40	80

A diagnosis of glandular fever was clinically probable but hæmatologically doubtful.

In connection with the possibility of false positive reactions, Barrett's (9) observations are important In studying 300 supposed normal sera, he obtained agglutination after guinea pig-kidney absorption in 15 cases at titres of 10 to 160 (1 case), in 10 subjects there was little or no absorption with ox cells—at the most a diminution in titre of one tube, and in most instances it seemed there had been partial but not the usual complete absorption with guinea pig kidney In 5 cases ox-cell absorption was good and the sera behaved as in glandular fever, in these subjects there was, however, no history of infection, they may or may not have been convalescents He felt that caution was needed in interpreting positive results and that lack of complete absorption with guinea pig kidney was not in itself diagnostic of glandular fever as it might occasionally be observed with normal sera

Barrett did not consider the test positive unless there was at least a 2-tube reduction in titre after absorption with ox cells, at low titres when both the normal and glandular fever antibody may be present together, this criterion is believed to be too stringent

Preparation of Guinea Pig-kidney Suspension and Heated Ox-cell Suspension [after Barrett (9)]

Guinea Pig-Kidney Suspension. The capsules and perirenal fat are stripped from at least two pairs of kidneys They are then washed well in running water. The tissue is transferred to a sieve partially immersed in normal saline, and rubbed with a pestle through the sieve The sieved kidney suspension is then autoclaved for 20 min at 15 lb. pressure and then rubbed twice more

The suspension
shed twice with
per cent phenol
in saline is added to the deposit, which is then resuspended in the

Ox-Cell Suspension Ox cells are washed well with normal saline, and a 30 per cent suspension of the cells in saline is autoclaved at 15 lb. pressure for 20 minutes. When cool, the suspension is strained through muslin and its packed cell volume estimated. The cell volume is adjusted to 20 per cent with saline and an equal volume of 1 per cent phenol-saline added to give a 10 per cent suspension. It is diluted to a 2 per cent suspension before use.

THE STAINING OF THICK BLOOD FILMS

The "thick" blood film has been extensively employed in the diagnosis of malaria and relapsing fever. By this technique a relatively large volume of blood may be scrutinized in a short time and parasites seen even if present in very small numbers. Field's technique is quick and generally satisfactory. A description of the method of preparation and use of the stains is followed by some notes as to its value.

Preparation of Field's Stains (14)

Field's stain "A" is made as follows :—

Methylene blue	1.3 g.
Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	12 6 g

The methylene blue and the disodium hydrogen phosphate are dissolved in 50 ml. distilled water The solution is then boiled and evaporated in a water bath almost to dryness in order to "polychrome" the dye 6 25 g potassium dihydrogen phosphate (KH_2PO_4) and 500 ml freshly boiled and still warm distilled water are then added After stirring to dissolve up the stain, the solution is set aside for twenty-four hours before being filtered. It should be filtered again before use

If Azure I is available, this may be combined with methylene blue in the proportion of 0.5 g. to 0 8 g methylene blue and dissolved directly in the phosphate buffer solution. No evaporation is necessary.

Field's stain "B" is made as follows :—

Eosin	1.3 g.
Disodium hydrogen phosphate (Na_2HPO_4 $12H_2O$)	12.6 g.
Potassium dihydrogen phosphate (KH_2PO_4) . .	6.25 g.
Distilled water	500 ml.

The phosphate salts are dissolved in freshly boiled and warm distilled water, and the dye then added. The solution should be filtered after standing for twenty-four hours

Making the Film. Thick films are made by spreading out with the aid of a needle or a corner of a slide a small drop of blood to cover an area of about 10–15 mm. in diameter. The slides must be clean and the film allowed to dry thoroughly at room temperature or at 37°C. before staining is attempted. A pause of thirty minutes is desirable ; absolutely fresh films, although apparently dry, may wash off in the stain.

Method of Staining. The dried but otherwise unfixed film is dipped into solution "A" for one to two seconds. It is then rinsed gently in neutral distilled water (or dilute phosphate buffer, *p*H 6.8 to 7 0) until the stain ceases to flow from the slide (five to ten seconds). Hæmoglobin is removed by these procedures.

The slide is then dipped into solution "B" for one to two seconds, followed by a rapid rinse (one to two seconds) in neutral distilled water. The excess water is shaken off the slide which is set upright to dry , it must not be blotted

The Relative Values of Thick and Thin Films

Thick films are extremely useful when parasites are scanty, but the identification of the different species is not easy ; mixed infections are often missed and there may be doubt as to the identification of any particular object However, an experienced observer may be able to find and recognize with certainty parasites even in badly stained thick films, whilst in a well stained film parasites should be easily recognized, even by beginners Five minutes spent in examining a thick film is equivalent to about one hour spent in traversing a thin film.

Study of *thin* films enables an exact diagnosis as to the species to be more easily made, *if* sufficient parasites can be

found. Seldom, if ever, should there be any doubt as to whether or not an object is a malarial parasite if the film has been well stained.

Thick films, if well stained, are useful where there is severe leucopenia. It is possible to perform differential counts (or at least to estimate the proportion of polymorphs present) much more rapidly and more accurately than in the case of thin films made from the same blood.

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APPENDIX 1

FORMULÆ AND PREPARATION OF DILUTING FLUIDS AND STAINING SOLUTIONS

Red Cell Diluting Fluid

A solution of 1 per cent formalin in 3 per cent sodium citrate. 10 ml. of neutral formalin (40 per cent formaldehyde) are added to 990 ml. of 3 per cent aqueous sodium citrate (see also p. 12).

The solution keeps well, preserves the discoidal shape of the red cells and does not cause hæmolysis or agglutination

Leucocyte Diluting Fluid

Two per cent acetic acid coloured with a trace of gentian violet or brilliant green (see also p. 43).

Platelet Counting Fluids

(1) *Lempert-Kristenson's Method*

Solution A. Sodium citrate 1 0 g
Mercuric chloride 0 002 g.
Brilliant cresyl blue 0 2 g

The reagents are dissolved in 100 ml. distilled water at 45°C.

Solution B Urea 20 g
Distilled water to 100 ml.

Equal volumes of *A* and *B* are mixed and filtered immediately before use (see also p. 111).

(2) *Method for Venous Blood* 1 ml of 0 5 per cent solution of brilliant cresyl blue in 0.85 per cent saline is added to 19 ml. of red cell counting fluid (formol citrate solution). The mixture should be made up freshly for each count and filtered immediately before use (see also p. 112).

Reticulocyte Counting Fluid

0.4 g. brilliant cresyl blue is dissolved in 100 ml. citrate-saline solution (one part 3 per cent sodium citrate, four parts 0.85 per cent sodium chloride). The solution must be filtered before use (*see also* p. 23).

The Preparation of an Artificial Hæmoglobin Standard [after Gibson and Harrison (8)]

11.61 g. chromium potassium sulphate [$CrK(SO_4)_2 12\ H_2O$, Analar], using crystals free from any signs of whitening due to efflorescence, 13.1 g. anhydrous cobaltous sulphate ($Co\ SO_4$) and 0.69 g potassium dichromate ($K_2Cr_2O_7$) are dissolved in 500 ml distilled water, 1.8 ml. N. sulphuric acid (H_2SO_4) added and the whole heated to boiling After boiling for one minute, the solution is cooled and the volume then made up to 1 litre with distilled water.

The cobaltous sulphate must be anhydrous About 30 g of $CoSO_4\ 7H_2O$ are heated for about two hours in a small porcelain dish placed in an oven at a temperature just below its melting point (90°C). The coarser particles should be broken up from time to time with a glass pestle The crystals should then be heated overnight in an electric muffle furnace kept at about 400°C. The product should be a uniform lilac powder This is transferred while still hot to a stoppered bottle As soon as it has cooled, 13 1 g are weighed out and dissolved in 80 ml of distilled water with the aid of heat As the anhydrous salt is hygroscopic, it can only be kept if sealed in glass tubes immediately after preparation

The standard is equivalent to 16.0 ± 0 2 g. hæmoglobin (based on iron determinations) when used as described on p. 30

Solutions for Supravital Staining

0.2 ml of a saturated solution of neutral red in absolute ethyl alcohol is added to 5 ml of absolute ethyl alcohol

0.02 ml and 0 05 ml. of a saturated solution of Janus green in absolute ethyl alcohol are then added to 1 5 ml volumes of the neutral red solution diluted as described above.

Slides are prepared using both the above solutions (*see also* p. 64).

Solutions for Peroxidase Staining

1. Fixative: absolute ethyl alcohol, nine parts; neutral formalin (40 per cent formaldehyde), one part.
2. A saturated aqueous solution of benzidine.
3. A saturated aqueous solution of sodium nitroprusside. Before use one part of (3) is added to 99 parts of (2).
4. A freshly made dilution of hydrogen peroxide ("10 vols"), 0.04 ml. are added to 10 ml. of distilled water (see also p. 68).

Solution for Staining Siderocytes

1. 2 per cent potassium ferrocyanide (freshly prepared).
2. N/5 HCl

Equal parts of 1 and 2 are mixed immediately before use (see also p. 73)

Appendix 2

FORMULÆ AND PREPARATION OF ANTICOAGULANT SOLUTIONS

FOR LABORATORY USE

Preparation of Tubes Containing Dried Oxalates [mixture of Heller and Paul (1)]

An aqueous solution is made containing 1.2 g. ammonium oxalate and 0.8 g. potassium oxalate per 100 ml. 0.5 ml. (containing 10 mg. of mixed salts) is delivered into test tubes which are then dried in an oven at a temperature not exceeding 80°C It is convenient to use tubes or bottles marked at the 5 ml. level 10 mg. of oxalate is sufficient to prevent the coagulation of 5 ml. of blood.

Preparation of Tubes or Bottles Containing Heparin

0 1 mg. heparin is usually sufficient to prevent the coagulation of 1 ml. of blood for at least twenty-four hours. For routine use it is, however, best to use twice this concentration. Heparin powder is dissolved in distilled water to make a solution of 4 mg per ml. 0.25 ml. of this solution is delivered into tubes or bottles previously marked at the 5 ml level. The containers are then placed in an incubator at 37°C. for about twenty-four hours, at the end of which time the heparin should be deposited as a dry almost invisible film

FOR BLOOD TRANSFUSION

Disodium citrate 16 6 g
Dextrose 25 g.
"Pyrogen free"* distilled water to 1000 ml.

* The presence of "pyrogens," i e dead bacteria or products of bacterial growth, in sterilized solutions or in the apparatus used for transfusions is generally held to be a potent cause of pyrexia and rigors developing after transfusions The content of "pyrogens" in water can be cut down to a

The above is distributed in 120 ml. volumes and autoclaved at 20 lb. for twenty minutes. 120 ml. contains sufficient anticoagulant for 420 ml. blood.

Blood preserved in the above solution and stored at the optimum temperature (about 4°C.) survives well after transfusion.† There is also the additional advantage that the dextrose and citrate solution, being a relatively acid mixture, may be autoclaved together without the dextrose being caramelized.

It is important that the autoclaves are properly used. The aim is to subject the article to be sterilized to moist heat at 120°C for at least 20 minutes; at this temperature even the most resistant bacterial spores are killed. To achieve this temperature, the autoclave must be emptied of air either by "blowing through" with steam or by means of some other device, and the steam allowed

tightened when the bottles are removed, if this is done, it is best that the bottles cool in the autoclave and that air is allowed to enter the autoclave through a cotton-wool filter. If the caps are tightened when the bottles are still hot, a vacuum within the bottles causes an undesirable inrush of air when the bottle is subsequently opened, and a serious likelihood of contamination. The efficiency of an autoclave can be simply gauged by its power of sterilizing garden soil. Other methods of assessment of their efficiency and further details as to the use of autoclaves in blood transfusion work are given by Spooner and Turnbull (2).

† The experimental evidence indicating that disodium citrate-glucose mixture is the best anticoagulant to use if blood is to be stored prior to transfusion, is given in the papers of Loutit, Mollison and Young (3), Loutit and Mollison (4), Loutit (5) and Gibson *et al* (6)

APPENDIX 3

PREPARATION OF USEFUL PIECES OF APPARATUS

Flask for the Defibrination of Blood

It is often convenient to use defibrinated blood as a source of serum. The blood (10 to 50 ml.) is delivered into a 100 ml. conical flask containing a central glass rod with a crooked irregular end (made by fusing small pieces of glass capillary to it) kept in position by a cotton wool plug (see Fig. 9). The flask is held by the neck and the contents rotated around the glass rod. The blood is usually successfully defibrinated and the fibrin entangled on the rod within five minutes. Little or no hæmolysis is caused by this procedure.

Glass Capillary Automatic Pipettes

Small automatic pipettes* made to deliver small volumes, say, 0.05–0.25 ml. of blood or serum, are of great use in clinical pathology for a variety of purposes, and in particular for making serial dilutions of serum, as in titrating iso-agglutinins. They are made of glass capillaries with the upper and lower ends slightly constricted and fixed in position with sealing wax in an outer glass tube (see Fig. 10).

These pipettes may be calibrated by making the capillary a little longer than will be required to deliver the required volume and gradually cutting it down until it delivers the correct amount—judged by delivering a series of volumes into a graduated centrifuge tube or 10 ml. measuring cylinder, or by weighing a series of volumes delivered into a previously weighed container. For making serial dilutions, exact calibration is unnecessary. Practice is required, however, before these pipettes can be effectively used, for in unpractised

* Details of the construction and use of automatic pipettes are given in Sir Almroth Wright's "Handbook of the Technique of the Test and Capillary Glass Tube," 1912, London.

FIG 9 FLASK FOR DEFIBRINATING 10–50 ML. OF BLOOD

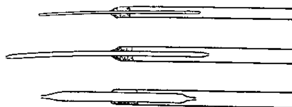


FIG. 10 GLASS CAPILLARY AUTOMATIC PIPETTES

hands fluid may be lost by being allowed to spill over into the outer tube. Their construction minimizes the risk, when making serial dilutions, of "carrying over" concentrated serum into tubes which should contain highly diluted serum, as may be done if a Pasteur pipette marked to deliver a certain volume is used for the titration.

APPENDIX 4

METHODS OF CLEANING SLIDES AND APPARATUS

New Slides. These should be placed in dichromate cleaning mixture (10 g. potassium dichromate dissolved in 100 ml. 25 per cent sulphuric acid) for at least forty-eight hours. They are then washed in running water, rinsed in distilled water and stored until used in 95 per cent alcohol. They should be dried with a clean linen cloth and carefully wiped free from dust before films are spread.

Dirty Slides. When discarded, they should be placed in 2 per cent lysol solution. They should then be washed under a tap and placed in a commercial detergent solution and boiled for twenty minutes. After a wash in tap water, they should be rinsed in 5 per cent HCl and then washed in hot running tap water. Finally, they should be rinsed in distilled water before being dried with a linen cloth.

Chemical Apparatus and Glassware The apparatus should be well washed in running warm water before being submerged in dichromate-sulphuric acid cleaning mixture for at least forty-eight hours. Then well washed in running water and finally rinsed in distilled water before being dried in an oven

Counting Chamber and Cover-slip. Immediately after use, they should be washed with warm water under the tap If greasy, they should be flooded with ether and dried with a clean linen cloth before the ether has had time to evaporate.

Blood Pipettes It is best to use a suction pump 2 per cent HCl is drawn through, then tap water This is followed by distilled water and then acetone (or alcohol followed by ether) Finally, air is sucked through until the pipette is dry.

Rubber Tubing Used in Blood Transfusion Work. A potent cause of pyrogenic reactions after transfusion is the

use of improperly cleaned tubing from which all traces of blood from a previous transfusion have not been removed. This risk can be minimized by washing through the tubing with running water as soon as a transfusion has been completed so as to prevent the possibility of blood drying within the tube. When returned to the laboratory, the whole apparatus should be dismantled and running water drawn through each piece of tubing until the efflux appears to be clear. A length of tape is then passed through with the aid of a stiff wire introducer and the tape then drawn backwards and forwards. By this means small pieces of adherent clot or fibrin can be removed. Running water should then be once more drawn through the tubing, and finally it should be rinsed with distilled water and allowed to drain and dry without delay.

THE USE AND STERILIZATION OF SYRINGES FOR HÆMATOLOGICAL PURPOSES

Two types of equipment may be used for obtaining blood by venipuncture, a dry syringe, preferably of the all-glass type, or a needle to which approximately 5 cm. of rubber tubing has been attached.

Syringes. Syringes of 10 ml. and 20 ml. capacity are convenient, bearing needles of 21 and 19 S.W.G. respectively. They may be sterilized with the piston and needle in position in glass tubes of a size slightly smaller than the diameter of the flange of the barrel (see Fig 11). It is convenient to place

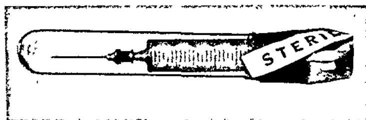


FIG 11 STERILIZED SYRINGE FOR THE COLLECTION OF 5–10 ML. BLOOD

a small piece of lint within the wrapping covering the open end of the tube; this piece of lint can serve as a sterile swab when blood is being withdrawn. Sterilization is affected by placing the wrapped syringe in a hot air oven at a temperature of 160°C for not less than one hour.

Syringes and needles should always be well washed through with water after use, before the blood within has had time to clot Before re-sterilization the syringe and piston must be thoroughly washed and cleaned *in hot soapy water and then* in running warm water and finally dried with a clean linen

cloth. The needle should be well cleaned with a pledget of wool on a stick or wire, as in a throat swab, and the lumen cleared with a wire stillette. Before the syringe is considered fit for use, the fit of the needle should be tested by forcibly sucking up water through the needle and noting how much air, if any, enters the syringe.

Needle and Rubber Tubing. Needles with olive mounts and of gauge 16, as used for giving blood transfusions, are suitable. Two inches or so of rubber tubing are attached to each needle. They should be placed in test tubes (see Fig. 12) and sterilized by autoclaving for twenty minutes at 20 lb. pressure. After removal from the autoclave, they

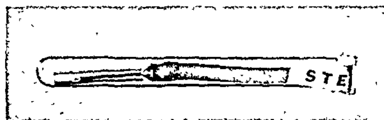


FIG 12. STERILIZED NEEDLE FOR THE COLLECTION OF 20–50 ML. BLOOD

should be "dried off" in an oven at a temperature not exceeding 100°C. With these needles 25–50 ml of blood or more may be rapidly withdrawn.

A great deal of useful information is contained in the M.R.C. War Memorandum on the "Sterilization, use and care of syringes" (7), a booklet every laboratory worker should possess.

Appendix 6

A NOTE ON RECORD KEEPING IN A HÆMATOLOGICAL LABORATORY

It is most important to keep serial records in the laboratory of the blood findings of every patient investigated. Only if the results of previous examinations are readily available, can an intelligent interest be taken in the progress of a case, and errors and discrepancies discovered. The filing card reproduced overleaf has been found useful. Spaces are available for the results of most of the more common investigations, and some room is left for recording the results of the less commonly performed tests. The cards are approximately 10×7 in. in size and printed on both sides. They are filed away arranged alphabetically according to the patients' names.

REFERENCES

(1) Heller, V. G., and Paul, H. (1934). "Changes in cell volume produced by varying concentrations of different anticoagulants" *J. Lab clin Med.*, 19, 777

(2) Spooner, E T C, and Turnbull, L. H (1942) "Notes on the use of [illegible] of blood trans-

(3) [illegible] Citric acid- [illegible] *Quart J exp.*

(4) [illegible]

(5) [illegible]

(6) [illegible] of plasma, by means of two isotopes of radioactive iron" *J clin Invest*, 26, 715

(7) "The sterilization, use and care of syringes" (1945) M R C War Memorandum No 15 H M Stationery Office, London.

(8) Gibson, Q H, and Harrison, D C. (1945) "An artificial standard for use in the estimation of hæmoglobin" *Biochem J*, 39, 490

NAME	CASE No	Laboratory No	Physician or Surgeon	Clinical Diagnosis

Date / Lab No	R B C per c mm ×10⁶	Hb % g	M C H γγ	M C H Conc %	P C V %	M C V cμ	Reticulocytes	W B C Per c mm	Myeloblasts	Promyelocytes	Myelocytes	Metamyelocytes (Young forms)	Metamyelocytes (Stab forms)	Segmented Polymorphs	Eosinophils	Basophils	Lymphocytes	Large mononuclears	Platelets per c mm ×10³	Other Data

FIG 13. FILING CARD FOR RECORDING SERIAL HEMATOLOGICAL DATA (natural size 10 × 7 in.)

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